SEAWATER/WASTEWATER PRODUCTION OF MICROALGAE-BASED BIOFUELS IN CLOSED LOOP TUBULUAR PHOTOBIOREACTORS

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ABSTRACT

Seawater/Wastewater Production of Microalgae-based Biofuels in Closed Loop Tubular Photobioreactors

Joshua Bradley Lowrey

The push for alternatives to petroleum fuels has forced researchers to look for highly productive, renewable, non-food resources. The advantages of using microalgae instead of traditional oil crops for biofuel production include high oil yields, rapid reproductive rates, and versatile growing requirements. In order to reduce the cost of producing microalgae based biofuels, wastewater has been used as a nutrient source instead of specialized plant nutrients. The purpose of this study was to compare the relative effectiveness of different combinations of microalgae strain and dairy wastewater for increasing biomass. The methods for monitoring growth included optical density, cell counting, biomass estimation by chlorophyll-a, and volatile suspended solids.

The analyses compared four concentrations of wastewater media as well as four strain treatments: *Chlorella vulgaris, Tetraselmis sp.,* mixed freshwater culture and mixed saltwater culture. Optimum wastewater concentrations for microalgae growth were approximately 0% and 25% for most strain treatments. The results of the wastewater treatments concluded that dairy wastewater could serve as an effective nutrient substitute for plant food at concentrations approximately 25%. *Chlorella vulgaris* and *Tetraselmis sp.* prevailed over the mixed cultures for biomass production. Nitrate was the most limiting nutrient and exhibited the greatest reductions, sometimes in excess of 90%. The regression equations derived from the volatile suspended solids data

achieved high R^2 values and determined that total nitrogen, ammonium, and nitrate were significant in the model. In those equations, increasing either ammonium or nitrate yielded an increase in volatile suspended solids. With regards to comparing biomass quantification methods, the two most useful and reliable biomass quantification methods were optical density and volatile suspended solids.

Keywords: microalgae, biofuels, biomass estimation, dairy wastewater, *Chlorella vulgaris, , Tetraselmis sp.,* mixed microalgae culture.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

1.1 Background Information

In July of 2008, world oil prices exceeded $140 per barrel, driving gas prices in the United States up well above $4 per gallon (EIA, 2010a). The magnitude of these events, along with many other factors, has generated tremendous incentives to research alternative fuels. Although petroleum alternatives have been under consideration for a few decades, the impact of inflated oil prices has left an unprecedented motivation to reduce dependence on petroleum (Broder and Connelly, 2010).

The search for alternative transportation fuels has many options varying in cost, potential, and technology required. While hybrid electric vehicles and all-electric vehicles are currently best poised to enter the mainstream market, biofuels are also accepted as a renewable option with great potential. One major attraction of biofuels for transportation is the opportunity to provide a fuel source without requiring large amounts of electricity from an already overloaded grid. In addition, one should consider that average electricity costs for the transportation sector have increased by over 25% from 2003 to 2006 (EIA, 2010b).

Biofuels, specifically comprised of biodiesel and bioethanol, have received increased demand, research, and investments in the last couple decades. The estimated subsidy by the United States to corn producers for fuel production is between

$820,000,000 and $1,368,000,000 per year (Koplow, 2006). These numbers depict the large investment being made to promote alternative fuels. While corn and soybeans dominate the production of biofuels in the U.S., research has suggested they perform poorly with regard to oil production efficiency (Chisti, 2007; EIA, 2009). One of the most promising alternatives to corn and soybeans for biofuel production is microalgae due to the extremely high growth rates and relative efficiency for oil production (Chisti, 2007).

As with any developing technology, utilizing microalgae for biofuel production still requires a substantial investment of financial and intellectual capital. Literature suggests that microalgae can offer tremendously high yields of oil per hectare and identifies various other advantages over traditional biofuel crops. However, the total cost of production has not yet become competitive with petroleum (Chisti, 2007; Mehlitz, 2009). In order for microalgae biofuels to become a viable alternative to petroleum, the production process must be streamlined to reduce the total cost.

1.2 Overall Industry Research Goal

Research is being done to bring down the cost of developing this energy source by reducing inputs into the process. Specifically, nutrient and carbon dioxide costs can be almost eliminated by utilizing existing waste streams. Additionally, the energy required for processing the algal biomass into biodiesel must be significantly reduced. Although there are theoretical methods available to address these challenges, the entire process must be streamlined through research (Pienkos and Darzins, 2009). By offsetting or minimizing these substantial costs through further research and ingenuity, microalgae can

become an economically competitive renewable fuel source. The overall research goal for this industry is to reduce the costs of producing microalgae and ultimately make microalgae a viable feedstock for biofuels.

1.3 Identification of Potential Research Goals

Reduce Production Costs:

1. Develop less expensive large-scale photobioreactors
2. Optimize natural or high efficiency lighting
3. Utilize wastewater as a nutrient resource

Reduce Processing Cost:

4. Develop lower-cost methods for extracting biomass from water
5. Develop lower-cost methods for extracting lipids from biomass

Maximize Biomass Productivity:

6. Increase lipid productivity of microalgae
7. Increase growth rates and total biomass of microalgae
8. Utilize genetic engineering to manipulate strain characteristics

Co-generate Biomass:

9. Utilize wastewater growing media for microalgae growth and reduce pollutants in wastewater
10. Sequester carbon dioxide from power plants, breweries, wineries, and other carbon dioxide emitters

1.4 Research Goals to be Investigated

Sub goals 3, 7, and 9 listed above were all investigated in this project. Because these sub goals are closely related, they were simultaneously investigated. The primary focus of the project encompasses utilizing wastewater as a nutrient source for growing microalgae, potentially increasing total biomass, as well as reducing pollutants in the wastewater. The purpose of the study is to examine the relative effectiveness of combinations of wastewater concentration and microalgae strain for increasing biomass growth. Additionally, various methods for quantifying microalgae biomass will be implemented and compared for their effectiveness.

1.5 Importance of Project

Consumption of bioethanol increased by 140% from 2000 to 2005 and biodiesel consumption increased by over 900% from 2001 to 2005 (EIA, 2007). Increases of this scale translate into tremendous pressure to provide greater production from biofuel feedstocks – which are the biological resources used to derive biofuels. Due to the limited oil production efficiency of corn and soybeans, as well as competition for food resources, meeting this increasing demand requires new feedstocks like microalgae (Chisti, 2007; Pienkos and Darzins, 2009).

When microalgae based biofuel production becomes competitive with petroleum prices, biodiesel will become a much more attractive option for transportation fuels. Among the proposed methods of achieving lower cost production for microalgae biofuels, there exists an opportunity for large industrial operations to benefit. Because these industries must meet water quality effluent regulations or air quality emissions

standards, they can look to microalgae for biological treatment of their waste streams. Utilizing microalgae for treatment of wastewater has been proven to remove large quantities of undesirable pollutants that would otherwise require costly treatment to remove (Hammounda et al., 1995; Woertz, 2007). Pairing microalgae production with these industrial operations can provide low cost inputs for biofuel production, reduce regulatory costs to the industrial partner, and improve the surrounding environmental quality.

The availability of more abundant and feasible alternative fuels in the transportation sector will generate competition with petroleum; thereby, driving down fuel costs for consumers. Additionally, emissions from biodiesel and bioethanol are significantly less than petroleum gasoline or diesel in almost all monitored constituents as shown in Figure 1 (USEPA, 2002). In the long term, society will have a more diverse and competitive fuel supply that will come at a reduced cost with fewer harmful emissions.

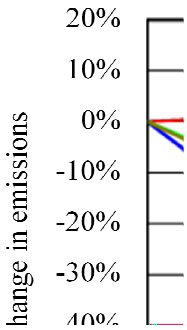

Figure 1. Percent change
oxides, PM = particulate
After USEPA, 2002.

1.6 General Approach

In order to exper
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Samples of microalgae were grown in lab-scale photobioreactors located indoors with independent controls and monitoring systems. Two strains of pure culture microalgae (one freshwater, one saltwater), and two mixed cultures (one freshwater, one saltwater) and four concentrations of wastewater were used. Measurements were taken on various indicators of microalgae growth, as well as a few primary nutrients during the growth period. The nutrients were observed to assess the wastewater remediation possibilities for agricultural waste streams and evaluate the impacts of nutrient availability on microalgae growth. The microalgae yields and nutrient changes were analyzed to determine the optimum growing conditions and the most successful microalgae treatment.

1.7 Specific Objectives

The major scope of the research questions for this study is identified by the following three specific objectives. These objectives will dictate the organization of this document.

1. Examine microalgae growth and estimate the relative effectiveness of combinations of dairy wastewater concentration and microalgae strains for maximizing biomass.
2. Observe nutrient uptake of microalgae at all treatments of wastewater concentration and strain selection for maximization of biomass and optimum wastewater remediation.
3. Compare four different recognized methods of biomass estimation and assess their relative effectiveness.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Throughout the last couple decades the public has been made increasingly aware of environmental problems that are becoming serious threats to human society. Climate change has emerged at the forefront of environmental problems and has received the most media attention as well as substantial international political discussion (Lindseth, 2004). The factor that distinguishes climate change from other environmental problems and generates more public interest is the multitude of social, political, and economic issues that are infused within the realm of climate change. Air pollution, water pollution, rainforest depletion, desertification, third-world resource shortages, nuclear proliferation, and petroleum shortages can all be related to climate change directly or indirectly (Botkin and Keller, 2007). While the roots of these problems vary depending on perspective, one underlying human necessity is a common denominator; energy (Hinrichs and Kleinbach, 2006).

The industrial revolution dramatically changed the methods by which humans derive their energy for all elements of society. Petroleum quickly dominated as an abundant, high energy product for electricity generation, transportation, and heating. The 1970s eventually illustrated the vulnerability of utilizing petroleum almost exclusively as an energy source. While environmentalists were advocating the air and water pollution problems from petroleum, the oil crisis of the early 1970s generated substantial incentive

for alternative energy initiatives (Schmidt, 2007). With the recent increase in oil prices another resurgence of funding for research has been invested in the development of alternative energies technology (Avato and Cooney, 2008).

The following review will explore the demand for biofuels and the current resources being used to generate them, microalgae and its potential for biofuel, classification and characteristics of microalgae, methods for large-scale cultivation of microalgae, and potential for research and application. The information should generate an understanding of the importance of investigating new resources for transportation fuel and the promise of utilizing microalgae as a biofuel feedstock.

2.2 Biofuels

Biofuel is energy recovered from organic matter known as biomass. Biomass can include forest products, agricultural residues, energy crops, animal residues or urban waste (Botkin and Keller, 2007; Hinrichs and Kleinbach, 2006). For the transportation sector, biofuels are usually limited to bioethanol and biodiesel because they are cleaner, easily transportable fuels that can operate on most existing automobile engine technology. While these fuels have been available for decades they are not in widespread use in the U.S. because of the low cost, high availability of petroleum fuels and low efficiencies of existing feedstocks (Hinrichs and Kleinbach, 2006). One major attribute that makes biofuels particularly enticing is their carbon neutrality, which means that there is zero net carbon being added to the atmosphere. Considering the rising concern about carbon dioxide and the effect of greenhouse gases on climate change, using biofuels presents an opportunity to reduce net carbon dioxide emissions.

Bioethanol (CH_3CH_2OH) is an alcohol that can be derived from fermenting biomass or gasification of plant matter. Bioethanol contains the same compound (C_2H_5OH) found in alcoholic beverages. The fermentation method for producing bioethanol is similar to common beer brewing techniques and can work for most biomass high in carbohydrates. Bioethanol is commonly blended into traditional gasoline to boost octane ratings and reduce air pollution, and requires only minor modifications to a traditional gasoline engine. While bioethanol can provide a viable alternative to pure petroleum, it is limited by a 34% lower energy yield than an equivalent volume of gasoline (USDOE, 2009; USDOE, 2010a).

Biodiesel is another liquid fuel made from renewable sources such as vegetable oils and used restaurant oils which can serve as an alternative to petroleum diesel. Biodiesel is also non-toxic and biodegradable, and burns with significantly less pollutants than its petroleum counterpart (USEPA, 2002). Today, biodiesel is commonly produced from restaurant waste and vegetable oils from plants like corn, soybeans, and sugarcane (USDOE, 2010a).

Increasing biofuel consumption has led to much greater production capacity in the United States (EIA, 2009). With this increased production capacity there is greater pressure on biofuel producers to find biomass resources (organic material to extract the oil from). Table 1 shows the increase in biodiesel and ethanol consumption from 2000-2005. Additionally, Figure 2 illustrates the increasing biodiesel production capacity relative to the increase in biodiesel consumption from 2001 to 2008. While production has managed to grow with demand, these biomass sources are beginning to constrict availability of vital food sources. The massive acreage of land required to grow these

biomass crops cannot possibly meet the requirements of the entire U.S. population's fuel needs (Chisti, 2007). To address this concern, various feedstocks – which are the biomass used for biofuel generation – are being considered for higher yields.

Table 1. U.S. motor fuels consumption, 2000-2005, million gallons per year. Source: After EIA, 2007.

	Gasoline	**Ethanol**	**Percent of gasoline pool**
2000	128,662	1,630	1.27
2001	129,312	1,770	1.37
2002	132,782	2,130	1.6
2003	134,089	2,800	2.09
2004	137,022	3,400	2.48
2005	136,949	3,904	2.85

	Diesel	**Biodiesel**	**Percent of diesel fuel pool**
2000	37,238	--	--
2001	38,155	9	0.02
2002	38,881	11	0.03
2003	40,856	18	0.04
2004	42,773	28	0.07
2005	43,180	91	0.21

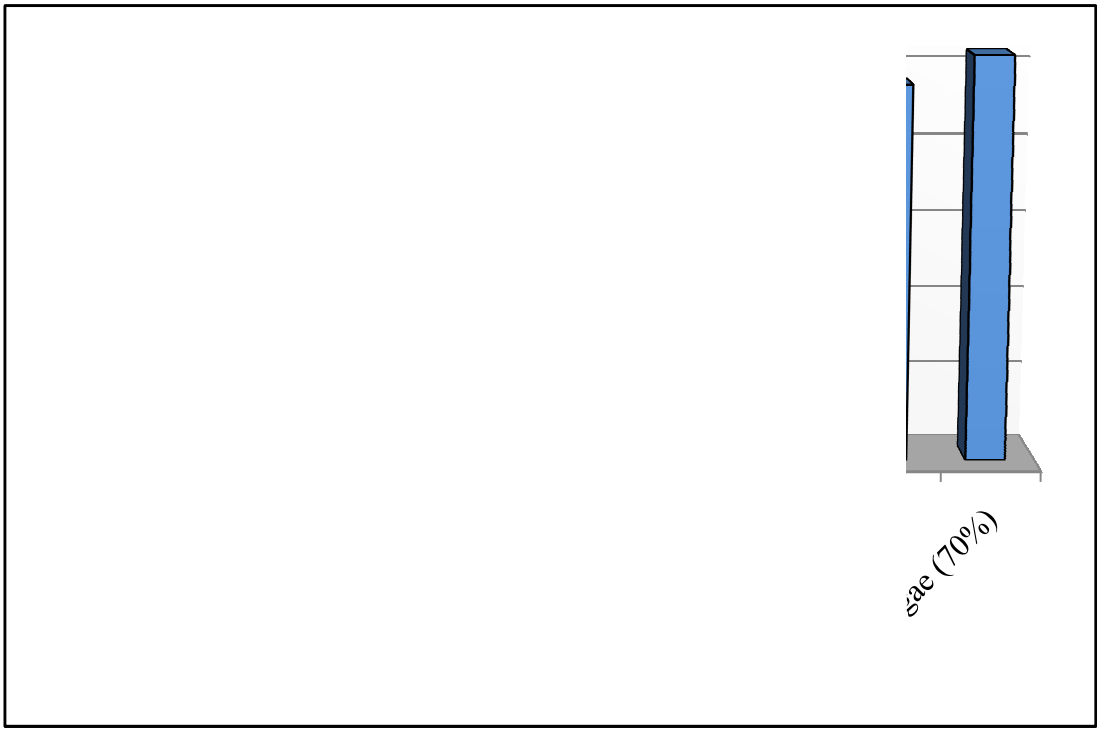
).

2.3 Biofuel Feedstocks

Biofuel feedstocks are the raw resources used to generate the sugar and oils necessary to create bioethanol and biodiesel (USDOE, 2009). The relatively new emergence of large scale biodiesel production has yet to overcome the limited feedstocks available for fuel. To date, soybeans are the primary feedstock for biodiesel production (EIA, 2009). Despite soybean dominance in biodiesel production, there are various alternatives that can be more efficient and cost effective. Figure 3 displays some major feedstocks and their respective annual yields in liters of oil per hectare. Although soybeans are the prevailing feedstock in the production market, the soybean yield significantly less oil per hectare than canola, jatropha, coconut, palm oil, and microalgae.

Two categories are provided for microalgae based on low (30%) or high (70%) oil contents.

A major concern for the feedstocks shown in Figure 3 is that most of the crops are also important food crops. For corn and soybeans specifically, as biodiesel production increases, food supply can be jeopordized. In 2008, the U.S. Department of Agriculture released a report citing increased biofuel consumption of the corn supply as part of the cause for a spike in food commodity prices in 2006 to 2007 (Trostle, 2008). This problem is a major argument for microalgae based biodiesel because microalgae are not a food crop, and do not require prime agricultural land for cultivation. Additionally, microalgae are expected to produce biodiesel at much greater efficiency than traditional crops even based on conservative estimations (Fig. 3).



2007.

2.4 Microalgae

2.4.1 Introduction to Microalgae

Microalgae are a diverse collection of photosynthetic, heterotrophic organisms that are known to have a tremendous reproduction rate (Bold and Wynne, 1978; Darley, 1982). These microscopic organisms can survive in harsh conditions across the earth's diverse landscapes; thus making them extremely abundant and diverse. Microalgae are also highly efficient photosynthizers due to their continuous suspension in water sources (Kirk, 1994). Akin to any photosynthetic organism, microalgae absorb CO_2 during photosynthesis, making them very valuable today for offsetting greenhouse gases (Pienkos and Darzins, 2009).

The primary composition of microalgae is proteins, carbohydrates, and lipids, which are the oils used for biodiesel production (Becker 2007). Their high lipid content relative to mass has proven to be particularly appealing for the production of biodiesel. Table 2 shows certain microalgae strains have been shown to have lipid content of upwards of 70% (Chisti, 2007). Compared to the average value of oil content by dry weight of corn which is somewhere between 3% and 8% (Heiniger and Dunphy, 2001), microalgae have tremendous oil content.

Table 2. Oil content of select microalgae. Source: After Chisti, 2007.

Microalga	Oil Content (% dry wt)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77
Tetraselmis sueica	15-23

2.4.2 Physical Properties

Algae are widely diverse groups of chlorophyll containing, mainly aquatic, eukaryotic organisms. They are different from plants by lacking stems, leaves, roots, and reproductive structures (Lee, 1999). Algae can be generally categorized into two major groups, microalgae and macroalgae. Macroalgae are multi-cellular organisms commonly known as seaweed that can reach sizes up to 50 meters (Darley, 1982). Microalgae are single celled organisms that normally survive in suspension in water (Darley, 1982; Tchobanoglous and Schroeder, 1987; MWH, 2005). Microalgae are microscopic cells measured in the range of micrometers (Bold and Wynne, 1978).

Eukaryotic algae cells are surrounded by a cell wall composed of polysaccharides. Additionally, surrounding the cell is the plasma membrane, a living structure responsible

for cross membrane flow of substances. Figure 4 shows that common plant cell organelles include a nucleus, chloroplasts, Golgi body, mitochondria. The flagellum (not shown in Figure 4) is an extended appendage that is used for locomotion (Lee, 1999).

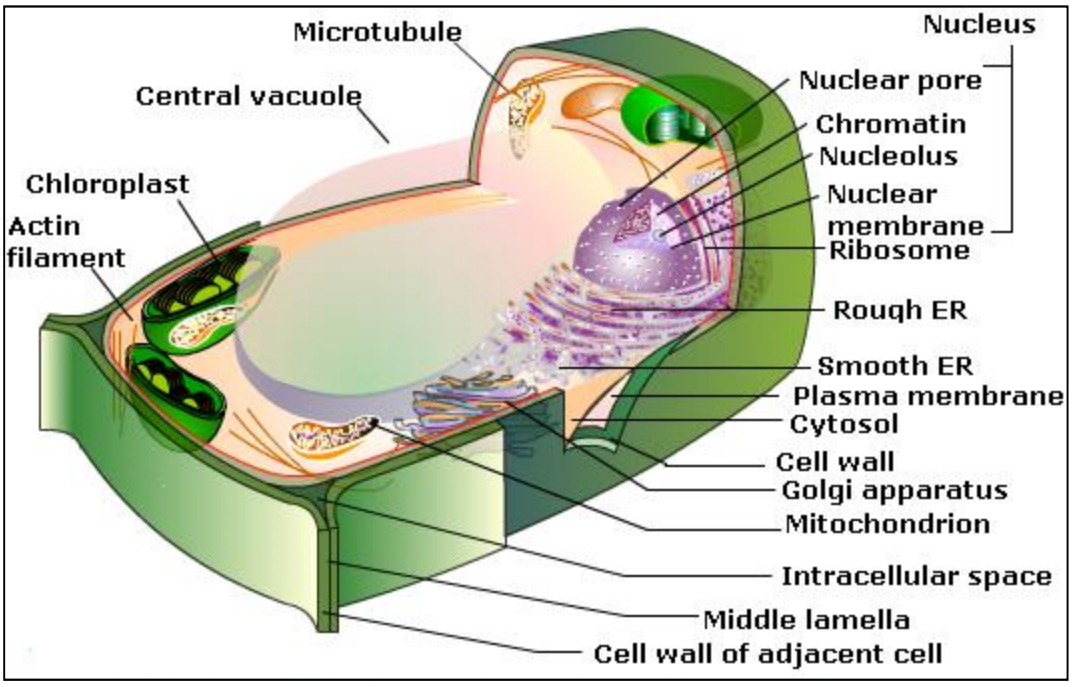


Figure 4. A typical plant cell. Source: After TutorVista, 2010.

As mentioned previously, algae are composed of proteins, carbohydrate, fats, and nucleic acids. Generally the proteins and carbohydrates are the majority, making algae cells an appropriate food source for many organisms (Becker, 2007). The lipid and fatty acid constituents of algae cells are suitable for conversion to biodiesel after extraction and transesterification (Vijayaraghavan and Hemanathan, 2009)

2.4.3 Classification

Historically speaking, there has been some uncertainty about the exact classification of algae. Traditionally, algae were classified as having both prokaryotic (lacking nucleus) and eukaryotic (with nucleus) domains (Bold and Wynne, 1978; South and Whittick, 1987). However, recent research suggests that the prokaryotic algae are more similar to bacteria than algae. Accordingly, newer definitions exclude prokaryotes from algae (Bhattacharya and Medlin, 1998). The most common breakdown of the eukaryotes includes four divisions: Rhodophycota, chromophycota, euglenophycota, and chlorophycota. Rhodophycota are red algae, chromophycota include all algae possessing chlorophylls *a* and *c* and lacking chlorophyll *b*, euglenophycota are similar to chromophycota yet disc or plate shaped, and chlorophycota are green algae (South and Whittick, 1987).

There are at least 100,000 discovered strains of microalgae and approximately 300 have been identified as candidates for biofuel production (Sheehan et al., 1998). These various strains can be freshwater or saltwater tolerant species. Similar to how the lipid composition of different strains varies, the protein and carbohydrate contents vary as well. These physiological differences are important for consideration of the biomass being utilized for biofuels. Various studies have been conducted that optimize the production of specific strains or characteristics within a strain with the goal of harvesting a specific product at optimum efficiencies. In one study, growing *Chlorella vulgaris* in low nitrogen environments showed increased calorific values – which are directly related to oil content (Illman et al., 2000).

2.5 Microalgae Growth Requirements

Microalgae have similar requirements for growth as any plant; light, water, CO_2, nutrients, and appropriate temperature. Research has been conducted on each input to microalgae growth to identify any possible opportunities to maximize biomass yield and reduce costs. Recent work is focused on utilizing waste streams – such as carbon dioxide emissions or nitrogen rich wastewater – as inputs into the microalgae growth process to reduce costs of generating biomass.

2.5.1 Light

Microalgae require both light and dark phases to conduct photosynthesis and respiration, respectively. Photosynthesis is conducted when chlorophyll captures light and converts water, carbon dioxide and minerals into energy rich sugars. Eqn. 2.1 illustrates photosynthesis (Smith and Smith, 2006)

$$6CO_2 + 12H_2O + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 + 6H_2O \qquad (2.1)$$

Photosynthesis in algae relies upon the conversion of light energy into chemical energy, most notably adenosine tripohosphate (ATP). This energy conversion is only possible within the photosynthetically active wavelength spectrum, 400 nanometers to 700 nanometers (Smith and Smith, 2006). Light absorption is generally reported in photons absorbed: micromoles per meter squared per second ($\mu mol \cdot m^{-2} \cdot s^{-1}$) or watts per square meter ($W \cdot m^{-2}$) (South and Whittick, 1987).

While light is essential for microalgae growth, a dark period is also required for respiration. The ratio of light phase to dark phase incident upon the cells is called the photoperiod (South and Whittick, 1987). The photoperiod is analogous to the mechanism for many circadian rhythms in living organisms (Darley, 1982). A balance between light and dark, similar to natural sun cycles, is required for cells to photosynthesize and metabolize carbon.

Providing light for microalgae in experimental conditions generates many questions of light intensity, photoperiod and light source. Indoor photobioreactors require artificial light and outdoor ponds need mixing for optimum light exposure in solution. With artificial light a concern arises over excessive light, inducing toxic conditions or diminished reproduction. However, some algae grow at higher rates under continuous light conditions due to the abundant energy influx (Darley, 1982). Information regarding light requirements for specific strains is scarce because algae have received little attention as compared to higher plants (South and Whittick, 1987).

2.5.2 Carbon Dioxide (CO_2)

CO_2 is required for plants to conduct photosynthesis, but also can be seen as an advantage of microalgae-based fuels that recycle CO_2 (Pienkos and Darzins, 2009). In photobioreactors, CO_2 can be used as both an input to photosynthesis, as well as a pH controller. As microalgae grow and uptake CO_2 within a closed system, the growth media exhibits an increasing pH. When CO_2 is added to the photobioreactor, it drives the formation of carbonic acid, thereby lowering the pH (Mehlitz, 2009). The following equations illustrate the role of carbonate chemistry on pH in water:

$$CO_{2\,(aq)} + H_2O \;<==>\; H_2CO_3 \quad (2.2)$$

$$H_2CO_3 \;<==>\; H^+ + HCO_3^- \quad (2.3)$$

$$HCO_3^- \;<==>\; H^+ + CO_3^{2-} \quad (2.4)$$

$$pH = -\log[H^+] \quad (2.5)$$

Aqueous carbon dioxide ($CO_{2\,(aq)}$), carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) are all in equilibrium depending on the concentration of hydrogen ions in solution [H^+]. Considering the preceding equations in continuity, all variables have a cascading effect on the others (Tchobanoglous and Schroeder, 1987). In a biological system, CO_2 and pH are variable, with a clear effect on one another. Simply adding CO_2 to a system increases the concentration of hydrogen ions in solution, thus reducing the pH.

2.5.3 Nutrients

Primary plant nutrients are known to be nitrogen (N), phosphorous (P), and potassium (K), and are all essential to growth of microalgae as outlined in Table 3. Aside from carbon, nitrogen is the most important nutrient for microalgae growth. Microalgae can assimilate both organic and inorganic nitrogen in the forms of urea ($(NH_2)_2CO$), nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+). Having a natural preference for organic urea (provided by animal excrement) as a nitrogen source, microalgae are well adapted to wastewater conditions (Darley, 1982). Ammonium is preferred over nitrate; and in some cases, high concentrations of ammonium will inhibit nitrate uptake (Darley, 1982). Algae are known to mainly participate in nitrogen fixation (reduction of $N_{2(gas)}$ to NH_3) and assimilation (conversion of NO_3^- and NH_4^+ to organic nitrogen), which

explains their affinity to NH_4^+ and NO_3^- (Barsanti and Gualtieri, 2006). Nitrogen limitation during microalgae growth has been shown to increase lipid content within the cells, while reducing growth rates (Illman et al., 2000).

Phosphorus, silicon, trace elements, and vitamins are also required for microalgal growth. Orthophosphate, PO_4^{-3}, is the only important phosphorus source for algae (Darley, 1982). Many primary and secondary plant nutrients can be provided through fertilizers and wastewater, which has very high nutrient contents. Growing microalgae in wastewater can prove to be beneficial for the metabolism of the microalgae cells, while simultaneously reducing the pollutants in the wastewater (Hammouda et al., 1995).

Table 3. Role of inorganic nutrients in algal metabolism. Source: After South and Whittick, 1987.

Element	Probable Functions
Nitrogen	Major metabolic importance as compounds
Phosphorous	Structural, energy transfer
Potassium	Osmotic regulation, pH control, protein conformation and stability
Calcium	Structural, enzyme activation, ion transport
Magnesium	Photosynthetic pigments, enzyme activation, ion transport, ribosomal stability
Sulfur	Active groups in enzymes and coenzymes, structural

2.5.4 Temperature

For effective growth of microalgae cultures an appropriate temperature range must be maintained depending upon the requirements of the strain. Every microalgae strain has a specific requirement for optimum temperature for maximum growth rates. Generally, temperatures between 15 – 25 ^{0}C are acceptable for microalgae (James, 1978). Algae exhibit normal temperature to biological activity relationship with activity increasing with temperature until an optimum temperature is reached. Above the optimum temperature, biological activity declines, sometimes abruptly, to zero (Darley, 1982).

While many experiments have provided optimum temperature ranges for known microalgae strains, determining the values independently is essential. With all other variables influencing growth held constant, temperature can be varied experimentally.

2.6 Growth Kinetics in Batch Culture

Growth kinetics is known as the relationship between the specific growth rate and the concentration of a substrate, or growth media (Kovarova-Kovar and Egli, 1998). Microalgae growth adheres to kinetics accepted for standard cellular growth. Cell growth can be conducted in either batch or continuous culture, which refers primarily to the availability of substrate. Batch growth refers to culturing cells in a closed vessel with an initial supply of growth medium that is not altered by further addition or removal. Alternatively, continuous culture refers to conditions where growth medium is continually supplied into a well-mixed culture and product is also continuously extracted (Schuler and Kargi, 2002). Lab-scale photobioreactors used for cultivation of microalgae follow the models of batch culture kinetics.

Commonly, populations of cells are quantified by direct cell enumeration, otherwise known as cell counting. Such growth measurements can be easily translated into kinetics models for batch culture. Cell kinetics is usually plotted as concentration of cells versus time. Figure 5 illustrates the standard sigmoidal curve associated with batch kinetics. The following phases are observed during batch cell culture (Schuler and Kargi, 2002):

1. **Lag phase:** The period during which the cell culture adapts to the new environment after being transferred. The lag in growth rate is attributable to the culture adapting to new substrate conditions and reorganizing molecular constituents to metabolize new substrate.
2. **Accelerated Growth:** After the culture adapts to new environmental conditions the growth rate begins to increase toward exponential growth.
3. **Exponential Growth:** The culture has fully adapted to the substrate conditions and is reproducing at the maximum growth rate. This phase is optimum to maintain production and commercial operations.
4. **Stationary Phase:** Cells begin to slow reproduction rates as substrate and light become less abundant and population reaches carrying capacity. Growth rates are either zero (no cell division) or equal to the death rate.
5. **Death Phase:** Substrate unavailability and competition result in cell death and infection from other microorganisms becomes more likely.

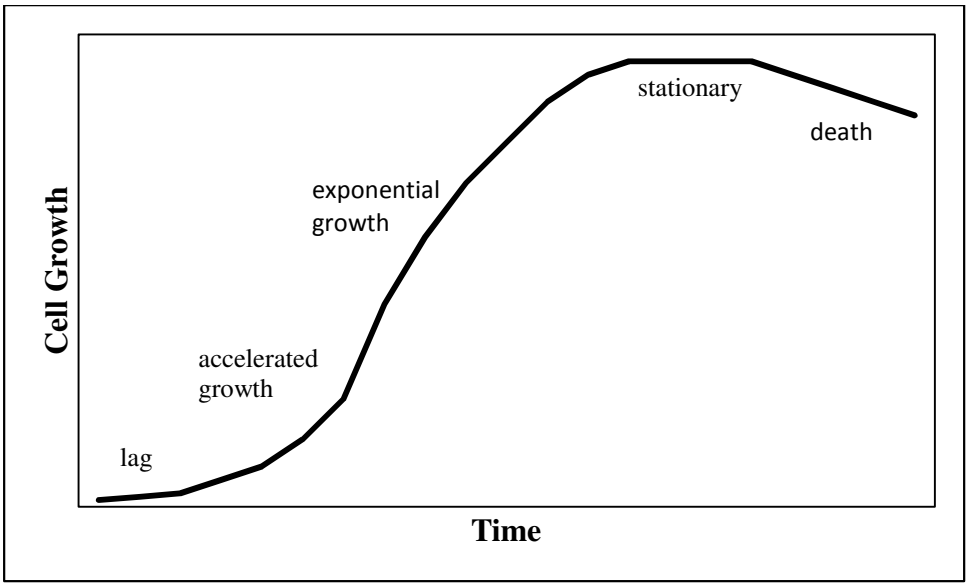


Figure 5. Standard growth phases of cell cultures. Source: After Schuler and Kargi, 2002.

2.7 Microalgae Culturing Techniques

Culturing is defined as growing isolated plant cells, tissues, and organs under axenic conditions to regenerate or propagate entire plants (Davey and Anthony, 2010). For proper inoculation of microalgae into a bioreactor, appropriately prepared cultures of the desired strain are required. In some cases, pure cultures can be ordered in bulk quantities from biological supply companies; alternatively, cultures can be prepared and maintained on site as well. Long-term maintenance of microalgae cultures requires sterile conditions as well as adequate light, nutrition, and temperature. A critical component of long-term maintenance is providing appropriate light and temperature for a dormant state, not optimum growth. This technique enables prolonged survival while minimizing potential for contamination. The primary concern when preparing cultures of

microalgae is preventing contamination from other microorganisms (Anderson, 2005; UTEX, 2010).

2.7.1 Sterilization Techniques

In order to maintain proper sterilization in a laboratory conducting microalgae culturing a proper aseptic protocol must be in place. Sterilization of laboratory supplies and equipment prevents unwanted organisms from creating potential variability in experimental results (Anderson, 2005). There are many options for sterilization of supplies and liquids such as dry heat, autoclave, chemical sterilization (via bleach or ethanol), filtration, and more advanced methods. Traditionally glassware, pipettes, and other handling tools must be sterilized in high heat or by autoclaving. For non-liquid materials, exposure to high heat (250 ^{O}C for 3-5 hours) is an acceptable method for sterilizing most laboratory supplies. The advantage of this method is that it can be conducted in a simple laboratory oven for relatively low cost. Liquids such as culture media and water must be sterilized through various means but commonly are autoclaved prior to use. Autoclaving is a process in which high steam pressure produces a sterilizing temperature on liquids without inducing evaporation (Anderson, 2005).

2.7.2 Culture Media

Culturing microalgae under experimental conditions usually requires culture media to provide the proper nutrition and optimum habitat for growth. Many acceptable culture media have been developed for individual strains to optimize growth or other desirable characteristics. Many of these media are formulated after extensive study of the nutritional requirements of microalgae strains while others model their natural environment. Culture media can be obtained through biological supply companies or

prepared in the laboratory (UTEX, 2010). Generally, media are composed of macronutrients, trace elements, and vitamins (Anderson, 2005).

2.7.3 Light and Temperature

Consideration of an appropriate light intensity and photoperiod for microalgae cultures can be critical to culture survival. While cultures require light for photosynthesis to survive, over-illumination is a common mistake made while culturing that can result in toxic conditions for algae cells. Standard light intensities for long-term preservation are 10-30 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} (Anderson, 2005). Photoperiod is the ratio of light exposure to dark that the culture is exposed to. The standard photoperiod for culture maintenance is 12 hours light and 12 hours dark (Anderson, 2005; UTEX, 2010).

Selecting the culturing temperature for microalgae depends on the native habitat of the strain, but can be generalized based on most strains. Major culture collections like University of Texas at Austin (UTEX) maintain temperature at 20 ^{o}C ± 2 ^{o}C. This temperature is sufficient to allow survival for both freshwater and marine strains without inducing significant evaporation (Anderson, 2005).

2.8 Microalgae Cultivation Methods

Cultivation, as opposed to culturing, is the process of growing the algae for production, usually on a larger scale for commercial applications (Becker, 1994). Cultivating microalgae for production of biodiesel requires large concentrations of microalgae biomass in controlled environmental conditions. Controlling the growth conditions allows optimized production for commercial-scale viability. Since naturally occurring microalgae blooms would not provide sufficient biomass for biodiesel

production needs, controlled growth environments have been developed. Raceway ponds were originally developed for wastewater treatment, and then adapted for microalgae growth. Photobioreactors were more recently developed to provide controlled growth of large quantities of microalgae with high levels of purity and sanitization (Carvalho et al., 2006).

2.8.1 Photobioreactors

Photobioreactors (also noted as PBRs) are closed systems in which all process inputs are controlled and microalgae are allowed to grow at optimum conditions. Photobioreactors are often arranged in series of transparent tubes. As shown in Figure 6b the arrays of tubes are positioned to maximize light penetration across all tubes (Chisti, 2007). When compared to raceway ponds, photobioreactors have shorter light paths, reduced contamination, better gas transfer, greater control, uniform temperature, and higher cell densities. Photobioreactors have many advantages over raceway ponds for cultivating microalgae, but the key distinction is the ability to maintain a closed system with no interference from the external environment (Carvalho et al., 2006; Chisti, 2007). Photobioreactors are preferred over raceway ponds when specific environmental conditions need to be controlled for applications such as a food processing where specific microalgae strains are being cultivated for food products.

2.8.2 Lab-scale Photobioreactors

Smaller photobioreactors used in laboratory settings are called lab-scale photobioreactors. Although they can vary in sizes and configurations, these generally have a transparent tube with a diameter less than 15 centimeters, and a length around 1 meter (Figure 6a). Lab-scale photobioreactors allow for controlled light, CO_2, nutrients,

and temperature. Photobioreactors of this size are used for experimental determination of optimum conditions to use in production-scale photobioreactors as well as growth vessels for innocullum into larger photobioreactors.

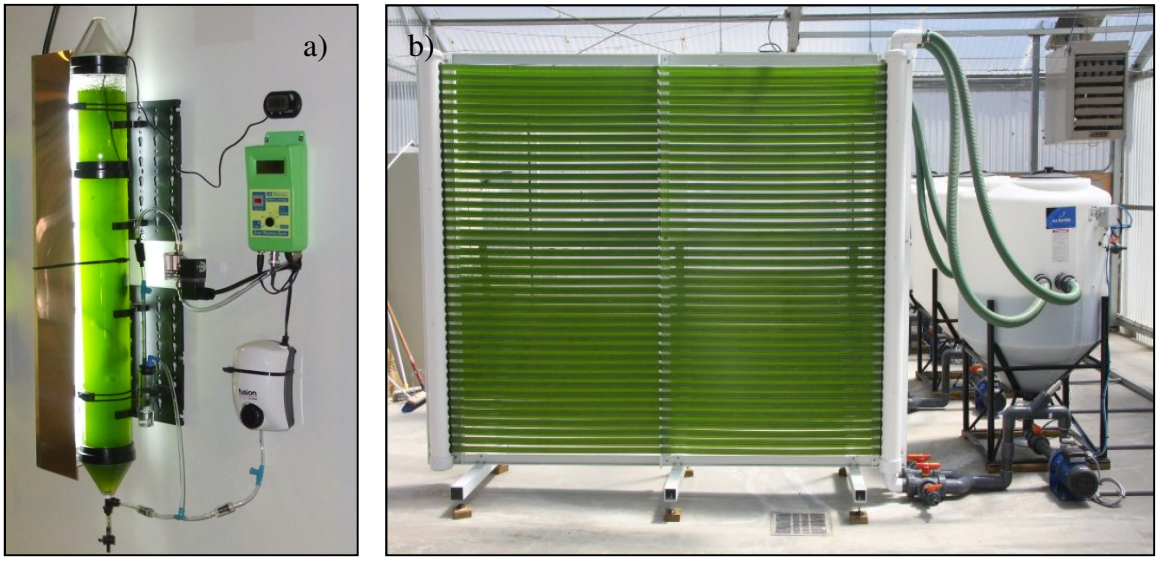


Figure 6. a) Cal Poly vertical lab-scale photobioreactor and b) production-scale horizontal photobioreactor.

2.8.3 Production-scale Photobioreactors

Large photobioreactors are used for production-scale and often consist of arrays of clear plastic tubes. Systems can have operating volumes ranging from hundreds to thousands of liters. Usually these systems consist of the tubular array, either vertical or horizontally aligned, and a storage receptacle that provides a respiration phase for the microalgal cells (Figure 6b). Controls are built in to regulate carbon dioxide and nutrients in the respiration phase before microalgae cells enter the tubular array. When the cells are in the array, they are exposed to light and conduct photosynthesis. These

systems are utilized for commercial biomass production, using experimentally determined optimum microalgae growing conditions (Carvalho et al., 2006).

2.8.4 Raceway Ponds

Raceway ponds are open waterways where microalgae are grown continuously in a simple outdoor system. The systems are similar to those used for wastewater treatment plants which also grow microorganisms for biological treatment of the wastewater. Seen in Figure 7, these systems are very large and are advantageous because of their utilization of natural light (Apt and Behrens, 1999). Raceway ponds tend to be less costly than photobioreactors due to minimal inputs and naturally provided light and carbon dioxide (Chisti, 2007). Many researchers believe raceway ponds are the only economically feasible way to cultivate microalgae on a large scale and that photobioreactors have not yet proven their superiority (Sheehan et al., 1998; Lee, 2001). However, research has proven that microalgae growth in raceway ponds is limited by surface area, effectively growing only in the top few millimeters of the water (Chen, 1996). The challenge of low light penetration is overcome by utilizing paddlewheels that continuously mix the water and allow better growth. Carbon dioxide is usually bubbled in from beneath at rates for optimum uptake by microalgae cells (Earthrise, 2009). Additionally, raceway ponds have demonstrated great water losses to evaporation (Chisti, 2007). While these systems have been extensively researched, they have proven to yield smaller biomass concentrations than photobioreactors and problems arise with contamination of microalgae strains.

Figure 7. Raceway ponds for microalgae production. Source: After Earthrise, 2009.

2.9 Microalgae Processing

One of the greatest challenges for generating cost effective biofuels from microalgae is processing the biomass into usable oils. The process of extracting the oils begins with isolating the microalgae cells from the growth media. Considering that an algae solution can be comprised of mostly water, this can be an intensive process. Figure 8 illustrates two identical samples and the large water content in a seemingly dense solution. The left sample was centrifuged to separate the microalgae from the water, and the right side was unaltered. The small mass at the bottom of the left tube is identical to the suspended microalgae seen in the tube on the right. The major methods for isolating the biomass are drying, centrifugation, and pressing the water out (Li et al., 2008). While many innovative alternatives exist for drying the biomass, the costs can become prohibitive.

Figure 8. Comparison of biomass in identical samples (left side centrifuged, right side unaltered)

Once the biomass is isolated from the water solution, the cells must be disrupted to obtain the valuable intracellular products. With regards to biofuel production from microalgae biomass, the lipids are the target product to extract. Lipids represent a class of organic compounds soluble in organic solvents, but insoluble in water. Included within the term "lipids" are glycerolipids, or fatty acids, which are useful for biofuel production (Hellebust and Craigie, 1978). Similar to biomass isolation, there are many techniques for this extraction. Many studies have been done analyzing lipid composition, quantity, and quality as well as comparing the extraction procedures (Huang et al., 2010; Hutton, 2009). The most common techniques are solvent extraction with an organic solvent (hexane, chloroform, methanol, acetone, etc.) or sonication , which ruptures cells with ultrasound waves (Hellebust and Craigie, 1978).

Once the lipids are extracted and homogenized, they must be processed into usable biodiesel. The traditional method is called transesterification, in which the triglycerides present in the lipids react with an added alcohol to form biodiesel and

glycerol. This is the common process used to form biodiesel from raw vegetable oils. There are also alternative methods of processing the triglycerides into biodiesel, but many are early in development and lack economical viability (Huang et al., 2010).

2.10 Challenges and Opportunities for Microalgae as a Biofuel Feedstock

For microalgae to become a mainstream biodiesel feedstock, the technology needs to be proven at production-scale. Many questions exist about the economic feasibility of microalgae because of the large production and processing costs, as well as uncertainty about the actual biodiesel yield (Sheehan et al., 1998). Although there has been significant research conducted on microalgae as a feedstock, the scale or process is usually limited for experimental purposes. Ultimately more work needs to be done on the whole system from cultivation to end-product.

Currently, research is emphasizing innovative ways to reduce costs of production and oil extraction. As discussed previously, there are many options and the potentially more cost-effective methods. With regards to reducing costs of producing the biomass, there is great potential to incorporate existing waste streams as valuable inputs to the growing process (Sheehan et al., 1998). Wastewater – both agricultural and domestic – has been utilized as a nutrient substitute for microalgae to metabolize. In many cases, wastewater provides an increase in biomass production compared to traditional nutrients. Additionally, the microalgae reduce pollutants in the effluent wastewater; thereby, providing a service of primary water treatment which can become a potential income source (Hammouda et al., 1995). Currently, research is conducted at municipal

wastewater treatment plants to partner microalgae growing in open raceway ponds, and simultaneously contribute to wastewater treatment (Hutton, 2009).

Carbon dioxide emissions have also been investigated as a means to utilize waste for microalgae growth. For example, industries such as power generation and ethanol fermentation have tremendous carbon dioxide emissions that are heavily fined by regulatory commissions. These emissions can be sequestered and bubbled through microalgae growing vessels for uptake during photosynthesis. This concept serves as evidence that growing microalgae as a biofuel feedstock can be partnered with other industries to reduce costs and streamline the production of biomass (Kadam, 1997).

2.11 Studies Using Wastewater Media for Microalgae Cultivation

As mentioned previously, microalgae can be utilized as a valuable tool for treating and mitigating wastewater from various industries with significant wastewater effluents. Some studies examine algae consortiums as a biological means for treating harsh industrial wastes due to algae's excellent viability under harsh conditions. In one particular study, microalgae strains *Chlorella* and *Scenedesmus* were used to treat toxic chlorinated organic compounds. The removal rates of certain compounds were found to achieve complete removal in 6-8 days while simultaneously generating algal biomass (Wu and Kosaric, 1991). Various other studies confirm the tremendous ability of algae to remove undesirable compounds from wastewater and thrive to generate valuable biomass (Hammouda et al., 1995; Tamer et al., 2006).

In addition to treating harsh industrial wastes, microalgae have been extensively used for nutrient removal in wastewater derived from agricultural or municipal sources.

While biological treatment of these wastes has long been used, interest has risen in quantifying the reduction of pollutants and monitoring the biomass generated for potential conversion into valuable products (Hammouda et al., 1994; Olguin, 2003; Munoz and Guieysse, 2006; Hutton, 2009). The idea of photosynthetic wastewater treatment dates back to wastewater treatment studies conducted by William J. Oswald in the 1950s. Oswald's studies explored using biological treatment in high rate ponds for the removal of specific compounds from wastewater (Oswald and Gotaas, 1957). For municipal wastewater, the removal of specific compounds is critical and heavily regulated by authorities. While conventional treatment considers removal of hazardous constituents for public health and water quality, microalgae serve to remove compounds that promote vegetative growth in natural water bodies. Studies have shown that removal of these compounds; namely ammonia, nitrate, and phosphorous, can reach nearly 100% removal using microalgae treatment (Martinez et al., 2000). A similar study using microalgae strains *Chlorella vulgaris* and *Scenedesmus rubescens* achieved approximately 90% removal rates in 9 days of ammonium, nitrate, and phosphate by growing the microalgae on municipal wastewater (Shi et al., 2007).

Agricultural wastewaters are also considered viable media for growth of microalgae due to their high nutrient content (Olguin, 2003). Experiments have been designed to explore the ability of agricultural wastewater to replace high cost specialized growth media for the production of microalgae (Wilkie and Mulbry, 2002). One specific study observed the growth rates of the cyanobacteria *Arthrospira platensis* and the nutrient uptake rates based on growth in agricultural wastewater. The experiment demonstrated effective growth rates and nutrient removal for select compounds (Lincoln

et al., 1996). In this study, biomass increased exponentially until 5 or 6 days and then began to decrease. Biomass values reported exceeded 1g/L and usually remained above 200 mg/L. The study also demonstrated substantial ammonia reductions with initial values above 100 mg/L and final values reported below 1 mg/L. Table 4 shows the nutrients observed after the full growth cycle.

Table 4. Final nutrient values after eight days. Source: After Lincoln et al., 1996.

Sample Number	NH_3-N (mg/L)	NO_3 -N (mg/L)	Total Phosphorus (mg/L)
1	<2.5	0.98	13.24
2	<2.5	1.40	16.01
3	<3.0	1.40	11.46
4	<3.0	0.28	17.28
5	<1.5	0.70	12.37
6	<3.0	1.40	11.04
7	<3.5	2.10	11.91
8	<3.0	1.40	14.40
control	44.5	2.80	22.93

The affect wastewater has on microalgae growth rates is also an important factor to consider for assessing the viability of using wastewater as a growth media. A study comparing the biomass production and nutrient removals of various filamentous cyanobacteria and diatoms verified that wastewater media does not hinder biomass productivity, and even enhances it (Kebede-westhead et al., 2003). Figure 9 illustrates the relationship between biomass production and wastewater loading rate. It is important to note that this study used periphyton, a mix of microorganisms, including algae, which inflates the biomass values as compared to pure microalgae. In Figure 9, the trend of both lines indicates that increasing loading rates of anaerobically digested dairy manure

results in increased biomass production. However, at greater loading rates, the production rate begins to level off.

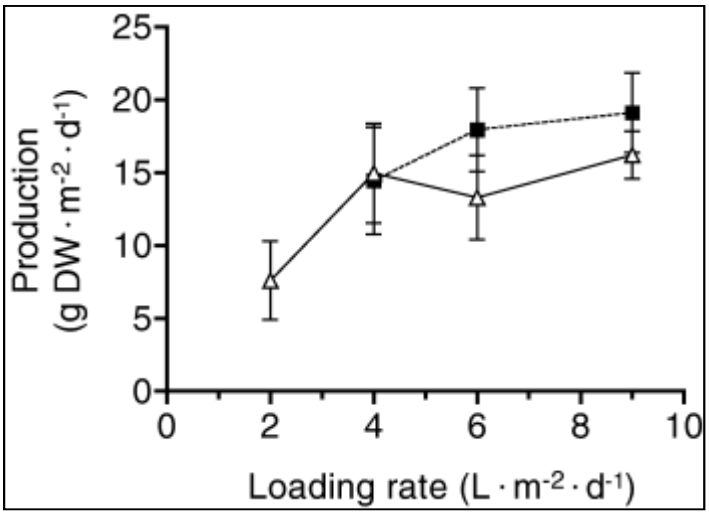


Figure 9. Algal production rate (g DW·m^{-2}·d^{-1}) under different loading rates of anaerobically digested flushed dairy manure wastewater and incident light (triangles, 270 µmol photons·m^{-2}·s^{-1}; squares, 390 µmol photons·m^{-2}·s^{-1}). Data points represent mean values of five to nine measurements, and error bars are SDs. Source: After Kebede-westhead et al., 2003.

2.12 Overview

Meeting the considerable demand for alternative energy sources demanded by economics, politics, and the environment requires many diverse solutions. Biofuels, and specifically biodiesel and bioethanol, provide a great alternative to contribute to society's transition away from petroleum reliance. While research has explored many feedstocks for biodiesel production, few have shown the promise that microalgae has for efficient oil production without threatening food supply. Utilizing an understanding of the advantages of various microalgae strains for specific purposes can help to optimize the efficiency of production. For example, a biodiesel operation would be interested in

cultivating microalgae strains with high oil content. Also, exploring options of growing conditions and photobioreactors can offset the costs that currently limit microalgae's potential as a biodiesel feedstock.

Microalgae are being explored for use as a biofuel resource, and as a biomass source for animal feed, composting, and extracting specific pharmaceutical compounds. The ability to effectively utilize every product of microalgae production assists in the cost effectiveness of the overall production schematic. There exists abundant research potential for not only improving the biodiesel production process, but altering carbohydrate or protein composition for animal feed, or even exploiting available valuable supplements found in microalgae biomass. The relatively small knowledge base in microalgae production presents an opportunity for assorted research topics in all stages of the production process.

CHAPTER III

MATERIALS AND METHODS

3.1 Microalgae Strains and Culture Maintenance

Pure cultures consisting of two freshwater and two saltwater microalgae strains were purchased from Carolina Biological Supply Company, Burlington, North Carolina. Freshwater strains were *Chlorella vulgaris* and *Scenedsemus sp.* while saltwater strains were *Tetraselmis sp.* and *Nannochloropsis sp.*. Contamination was so prevalent in the *Scenedesmus sp.* and *Nannochloropsis sp.* cultures that those strains were reclassified as mixed culture. The resulting strain treatments were freshwater *Chlorella vulgaris*, mixed freshwater culture, saltwater *Tetraselmis sp.*, and mixed saltwater culture. These microalgae strain treatments were then carefully scaled up into larger volumes more appropriate for inoculating the lab-scale photobioreactors. Initially, the pure culture was transferred into a clean 250 mL Erlenmeyer flask, and then plant nutrients were added from a prepared medium provided by Schultz Company. The flask was then brought to volume with distilled water or an Instant Ocean Sea Salt mix, depending on the strain. The 250 mL culture flasks were stored at approximately 20 ^{O}C under a fluorescent light (34W/2650 lumen/4100K) with a 12:12 light cycle. There was no agitation or CO_2 provided until scale-up to larger flasks.

Figure 10. Microalgae cultures for long-term storage.

Once the 250 mL culture was sufficiently dense (1-2 weeks), based on visual assessment, the culture was transferred to a sanitized 1 liter Erlenmeyer flask and brought to volume with distilled or saltwater. All flasks were enclosed with a rubber two-hole stopper to provide an input for air/carbon dioxide mixture, and a vent. An air/CO_2 mixture was provided for the 1 liter flasks by an air pump attached to an air stone in the flask that was run continuously. CO_2 was dosed into the culture for 5 minutes daily. Eventually the cultures were transferred to sanitized 2 liter Erlenmeyer flasks prior to inoculation into the lab-scale photobioreactors. All cultures for the four microalgae strain treatments were maintained at approximately 20 ^{o}C under fluorescent light (34W/2650 lumen/4100K) (Fig. 10). Cultures were required to reach sufficient density, 40% transmittance at 665 nm, prior to inoculation for experiments. For inoculation, 225 mL (10% of total PBR volume) of cultured microalgae was aseptically transferred into the lab-scale photobioreactors. The remaining volume of the PBR was filled with a wastewater and distilled or saltwater ratio. These specific volumes are discussed further in section 3.6.

3.2 Nutrients and Dairy Wastewater

Plant nutrition was provided primarily by dairy wastewater collected from the 400-head dairy facility at Cal Poly San Luis Obispo shown in Figure 11. The dairy wastewater was derived from washout of excrement in the stalls and was screened for most large solids. After screening, the wastewater was transferred to large open storage ponds, as seen in Figure 12. While in the pond, the wastewater undergoes partial biological digestion under aerobic and anaerobic conditions (Woertz, 2007). For this study, wastewater was collected in Summer of 2009, and processed through a continuous centrifuge to remove larger particulates. After processing, the wastewater was stored indoors in a 50 gallon plastic drum at the same location as the photobioreactors.

Figure 11. Dairy stalls at Cal Poly San Luis Obispo.

Figure 12. Aerobic dairy wastewater lagoon.

The biochemical composition of this wastewater was uncertain but it was assumed to have very high concentration of primary and secondary plant nutrients. Additionally, such a dynamic media was likely comprised of continuously changing nutrients, and one sample may not be representative of the experimental conditions. However, Table 5 provides a paraphrased snapshot of the same dairy wastewater collected at an earlier date in 2007 for Ian Woertz's research.

Table 5. Diluted dairy wastewater composition. Source: After Woertz, 2007.

Wastewater Characteristics	**25% Wastewater**	**10% Wastewater**
Ammonia (NH_4-N)	30.5	16.3
Nitrate (NO_3-N)	0.01	0.05
Nitrite (NO_2-N)	<0.01	0.04
Total Nitrogen	81.0	36.6
Phosphate (PO_4^{3-}P)	2.55	1.8

For the control treatments of the experiment, Schultz Plant Food was used as a nutrition source for the microalgae. This plant food was selected to represent basic fertilizers as opposed to the nutrition provided from wastewater. Schultz Plant Food was a general purpose plant food intended to optimize growth of most plants. Table 6 illustrates the N-P-K ratio for Schultz Plant Food which is 10-15-10. For each liter of media, 0.65 mL of plant food was added, resulting in a total volume of 1.45 mL for the lab-scale photobioreactors.

Table 6. Manufacturer reported nutrient composition of Schultz Plant Food.

Analysis	
Total Nitrogen 1.6% Ammoniacal N 0.2% Nitrate N 8.2% Urea N	10%
Available Phosphate (P_2O_5)	15%
Soluble Potash (K_2O)	10%
Iron (Fe)	0.10%
Manganese (Mn)	0.05%
Zinc (Zn)	0.05%

3.3 Lab-scale Photobioreactors

The lab-scale photobioreactors were the clear plastic vessels in which the microalgae were cultivated under experimental conditions. The photobioreactors from Aqua-Medic Company in Fort Collins, CO provided a 2.25 liter tubular plastic growing chamber with enclosed fluorescent lighting (6700K, 18W, 1300 lm). Air was pumped from beneath for respiration and agitation, pH regulators, carbon dioxide controls, and temperature monitoring and heating were in place for controlled environmental

conditions (Fig. 13). Each photobioreactor regulated light, temperature, carbon dioxide, and pH for experimental consistency. The fluorescent light was operated on a 16:8 light:dark photoperiod. The location of the photobioreactors offered almost no natural sunlight. The rating of the fluorescent bulbs is intended to provide bright outdoor conditions (65 $W \cdot m^{-2}$) for the reactor. The air pump, a Fusion Quiet Power 400, was run continuously, and the temperature and CO_2 were regulated by the Coralife digital thermometer and Milwaukee SMS122 pH computer, respectively. There were six photobioreactors in total, which were continuously operated as experimental replicates. The photobioreactors were located indoors at California Polytechnic State University in San Luis Obispo, CA.

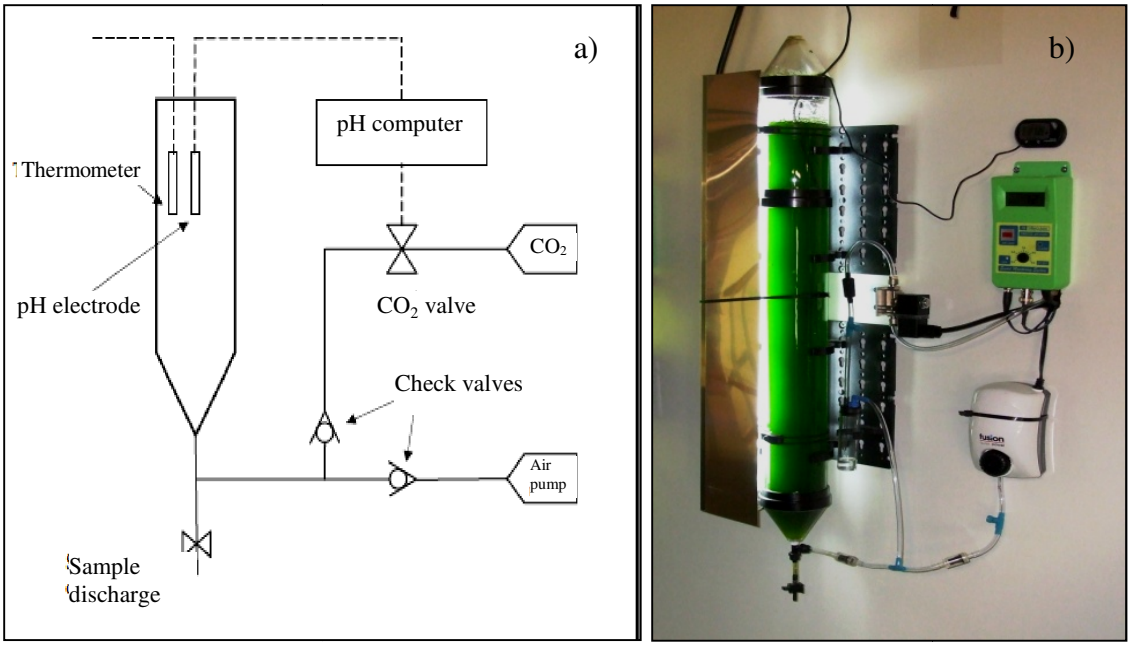


Figure 13. a) Schematic view of the photobioreactor setup (Source: After Mehlitz, 2009) and b) a vertical tubular photobioreactor on Cal Poly campus.

3.4 Spectrophotometer

A Hach DR3800 UV/Vis spectrophotometer as seen in Figure 15 was used to monitor algal biomass and nutrients using methods adapted from Standard Methods for Examination of Water and Wastewater (APHA, 1998). A spectrophotometer measures the absorbance of light at specific wavelengths by directly measuring the light before and after it passes through a sample. Utilizing the Beer-Lamberts Law – which defines the physical laws for spectroscopy – and the values obtained from the spectrophotometer, calculations can be made to get data for algal biomass and nutrient composition. The Beer-Lambert Law states:

$$A = -\log \frac{I_T}{I_O} = \varepsilon dc \qquad (3.1)$$

Absorbance (A) is dependent upon the initial (incident) light intensity (I_o) and the final (transmitted) light intensity (I_T). Alternatively, absorbance can be related to the molar absorptivity of the sample (ε), the path length (d), and the concentration of the sample in moles per liter (c) (Swinehart, 1962).

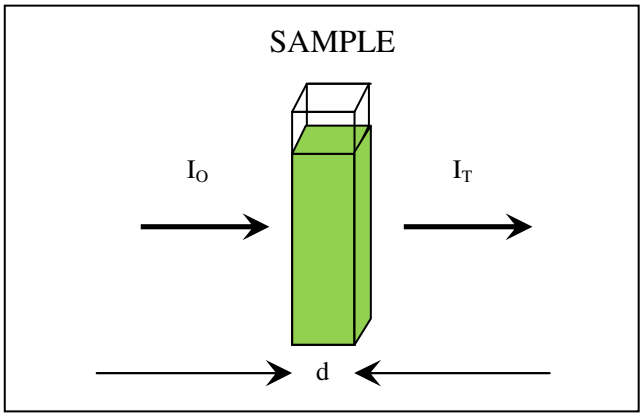


Figure 14. Visualization of Beer-Lambert principle.

Figure 15. Hach DR3800 spectrophotometer.

For the purpose of monitoring microalgae, the specific wavelength of 665 nm was selected to capture the chlorophyll pigments present in the sample. This value was selected as an ideal wavelength for estimating the chlorophyll-a content, which is present in the microalgae under investigation (Hellebust and Craigie, 1978). Although this measurement is not a direct measurement of microalgae density, it acts as an indicator by using optical density. Simply, as the sample becomes more dense (darker color) the absorbance of the sample increases correspondingly. The justification for this method is the high correlation between optical density and other biomass quantification methods seen later in Table 31.

The spectrophotometer was also used for was monitoring select nutrients in the growth media which was conducted using Hach Test in Tube Plus (TNT+) kits. These kits are prepared standard methods for measuring specific elements in a sample in the absence of advanced laboratory facilities. In practice, the TNT+ kits require brief in-lab procedures followed by analysis by the corresponding Hach spectrophotometer. The nutrient monitoring methods will be further discussed in section 3.12.

3.5 Digital Microscope and Hemocytometer

A Motic BA310 microscope equipped with a digital camera was used for microscopic analysis of sample purity and cell counting. The microscope was equipped with 4X, 10X, 20X, 40X, and 100X phase contrast objectives, and a 10X eyepiece allowing for 1000X maximum magnification. The phase contrast objectives enable viewing of live biological samples without requiring a stain, which would kill the microorganisms. The digital camera allows for live-feed video and images to advanced

imaging software. Cell counting was conducted using a hemocytometer and phase contrast objectives for viewing living organisms. A hemocytometer is a specialized microscope slide with a small etched grid on the surface illustrated by Figure 17. The grid is enclosed by surrounding walls to form a counting chamber. The counting chamber has a known volume to enable users to calculate cells per volume (APHA, 1998).

Figure 16. a) Motic BA310 microscope. Source: After Motic, 2010.

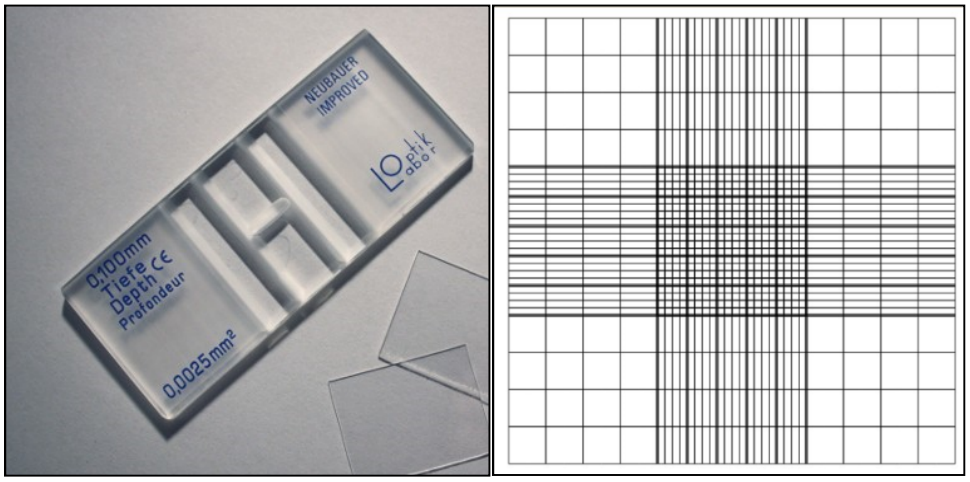


Figure 17. a) Hemocytometer and b) hemocytometer counting grid. Source: After Kim, 2010.

3.6 Experimental Design, Data Collection and Analyses

The experimental design used in this study was a split-plot design consisting of two experimental factors, microalgae strain and wastewater concentration. Table 7 shows how microalgae strains were randomly assigned to main plots and wastewater concentration was randomly assigned to sub plots within each main plot. The two independent variables for the experiment are microalgae strain and wastewater concentration. Each independent variable had four levels; four microalgae strain treatments (two freshwater and two saltwater), and four specific concentrations of wastewater (0%, 10%, 25%, 33%). The microalgae consisted of two pure cultures, *Chlorella vulgaris* (freshwater) and *Tetraselmis sp.* (saltwater), and two mixed cultures (one freshwater and one saltwater). The mixed cultures were generated from original cultures of freshwater *Scenedesmus sp.* and saltwater *Nannochloropsis sp*., and eventually became a medley of microorganisms. The 0% wastewater concentration served as an experimental control with recommended nutrient dosage from Schultz Plant food. Based on the four levels of two independent variables, there were 16 total treatments. Each treatment consisted of six replicates and underwent a 7 day growth period.

Table 7. Experimental factors used in the split-plot design for this study.

Main Plots (Microalgae Strain)	Sub Plots (Wastewater Concentration)
Chlorella vulgaris	0%
Tetraselmis sp.	10%
Mixed Freshwater (*Scenedesmus sp.)*	25%
Mixed Saltwater (*Nannochloropsis sp.*)	33%

Due to spatial and financial limitations, sample size was limited to six replicates. Data was collected on: cell counts, algal biomass (by chlorophyll-a), optical density, total suspended solids/volatile solids, nutrients (total nitrogen, ammonia, nitrite, nitrate, total phosphorous), temperature, and pH. Temperature and pH were monitored daily; cell counts, optical density and algal biomass every other day; and solids and nutrients were measured for initial and final values (Table 8).

Table 8. Schedule of methods measured for each treatment. (OD: Optical Density, TSS: Total Suspended Solids, VSS: Volatile Suspended Solids).

Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Temp	Temp	Temp	Temp	Temp	Temp	Temp	Temp
pH	pH	pH	pH	pH	pH	pH	pH
Cell Count		Cell Count		Cell Count		Cell Count	Cell Count
Chlorophyll		Chlorophyll		Chlorophyll		Chlorophyll	Chlorophyll
OD		OD		OD		OD	OD
TSS/VSS							TSS/VSS
Nutrients							Nutrients

All experimental microalgae growth occurred in closed systems in 2.25 liter tubular photobioreactors purchased from Aqua Medic GmbH. The photobioreactors were inoculated with 225 mL (10% total volume) of microalgae cultures with 40% transmittance at 665 nm. The designated volume of wastewater (0%, 10%, 25%, or 33%) was then added along with 1.45 mL of plant food for the control treatments. The

remaining volume was filled with distilled water or an Instant Ocean saltwater mix, depending on strain. The temperature and pH were set at specific levels to optimize growth of each microalgae strain, based on existing literature presented in Table 9. The mixed cultures were maintained at the conditions of their corresponding initial pure culture inoculum. Light intensity was maintained constant using a fluorescent bulb as described in section 3.3. Ideally, the only changes in environmental conditions were due to the various levels of the independent variables. After the 7 days, treatments were completed, and the photobioreactors were disassembled and sterilized for the next treatment period. To sterilize the plastic components of a photobioreactor a phosphoric acid-based Star San general sanitizer was used followed by rinsing with distilled water.

Table 9. Optimum growing conditions maintained for select microalgae strains.

	Strain	Temperature, °C	pH	Characteristics
Freshwater	*Chlorella vulgaris*	32	6.5	Nutrient removal, high growth, commonly used (Mayo, 1997; Olguin, 2003)
	Mixed culture *(Scenedesmus sp.)*	30	Tolerant	Nutrient removal, commonly used (Martinez et al, 2000)
Saltwater	*Tetraselmis sp.*	19-25	Tolerant	High lipid content, wastewater tolerant (Griffith et al., 2009)
	Mixed culture *(Nannochloropsis sp).*	25	7.8	Nitrogen and phosphorus removal (Zittelli, 1999)

* pH tolerant strains were maintained at pH 7-8

The measured outcomes of each independent variable were analyzed both graphically and statistically. During the graphical analysis, the mean values for each treatment were calculated, after removing outliers or impossible observations. Error bars were presented using the standard error of the mean. All correlations were developed by generating the Pearson's correlation coefficient and the corresponding p-value for determination of significance. Correlation was used for comparison of microalgae quantification methods as well as to confirm the relationship between the uptake of nutrients.

3.7 Statistical Analysis Techniques

Hypothesis testing was used to make statistically significant conclusions about the data using a significance level of 0.05. Statistical techniques were employed to test the following hypotheses regarding quantifying and comparing microalgae growth for the various treatments:

Hypothesis testing for strain treatments:

Null hypothesis (H_o): $\mu_C = \mu_{MF} = \mu_{MS} = \mu_T$ Alternative (H_a): at least one differs

Hypothesis testing for wastewater concentration:

Null hypothesis (H_o): $\mu_0 = \mu_{10} = \mu_{25} = \mu_{33}$ Alternative (H_a): at least one differs

The hypotheses used for comparing the microalgae quantification methods for their relative efficacy are:

Hypothesis testing for method comparisons:

Null hypothesis (H_o): there is no correlation between the methods ($\rho = 0$)

Alternative (H_a): there is a correlation between the methods ($\rho \neq 0$)

Using SAS 9.0 and Minitab 15, split plot analysis of variance (ANOVA) was used to determine the significance of the differences between treatments in the experiment. An ANOVA was conducted for each microalgae quantification method separately to determine the significance of each treatment level on growth. With wastewater concentration and strain selected as the main effects, Tukey comparisons were generated for significant effects. For significant main effects, the comparisons were made for the differences between the four levels of each main effect. If interactions were found to be significant, further analysis of specific combinations of wastewater and microalgae strain could be statistically analyzed.

Multiple linear regression was also conducted on the data collected for nutrient uptake. The regression analysis was also separated by microalgae quantification method, with four total regression equations being derived. The design of the regression analysis was to set microalgae growth as the dependent variable with strain and initial nutrient concentrations as the predictor variables. The models were built using stepwise

backward elimination to only include significant predictor variables in each model. Coefficients of determination (R^2 values) were reported for each regression equation.

3.8 Optical Density Measurements

Optical density is a common measurement used to simply quantify the ability of light to pass through a sample at a specific wavelength. The concept was derived from the Beer-Lambert Law, and utilizes the absorbance values as an optical indicator of sample density. In practice, the well-mixed sample is placed in a clean cuvette and absorbance is measured in a spectrophotometer at 665 nm. Usually, the darker the sample is visually, the greater the algal density. Figure 18 shows two cuvettes filled with two samples where the sample on the left is much darker, and therefore, more dense. Optical density is an excellent indicator of microalgae density in a sample, but suffers from interferences with turbidity and cannot distinguish between strains of microorganisms.

Figure 18. Visual comparison of optical density in cuvettes.

3.9 Cell Counting

While there are many deviations on the method used to manually count cells using a hemocytometer, the basic concept is universal. In general, a small amount of sample is placed on a specialized microscope slide with a known area and depth in the counting chamber. The counting chamber is the small space where the sample exists between the top of the slide and the cover slip. On the counting chamber is a finely etched grid that defines a precise surface area for counting to occur. When looking at the slide through the microscope, the cells in a few of the squares are counted, and the number is extrapolated into cells per volume based on the known spatial parameters of the counting chamber (APHA, 1998). This method allows estimated figures to be obtained about how many cells exist of a specific specimen. Samples do not need to be stained when viewing with a microscope equipped with phase-contrast objectives. The cell count method yields units of cells per milliliter and can be used to determine doubling time. A detailed procedure for this method was provided in Appendix A.

Cell counts are excellent measures of sample purity and density, but are very time intensive and do not provide mass-based units. Despite the time requirement, it is essential to monitor contamination of experimental strains. Additionally, the cellular units and doubling time are very instrumental in conveying data in a clear and tangible way. Division rates are calculated using two cell counts with a known time elapsed between the measurements. The calculation for cell division rate is as follows:

$$k = \log (N_1/N_0) (3.222/t) \quad (3.2)$$

k = divisions per day, N_0 = initial cell count, N_1 = final cell count, t = number of days

3.10 Algal Biomass Estimation by Chlorophyll-a Determination

An accepted method for estimation of algal biomass is based on the measurement of chlorophyll-a content of the sample. Initially, the sample must be isolated from the water by centrifugation or filtering. The isolated biomass must then be separated for extraction of chlorophyll. The chlorophyll extraction is conducted by macerating the sample in a tissue grinder with an aqueous acetone solution. After isolating the biomass from water and extracting the chlorophyll, chlorophyll-a can be estimated using a spectrophotometer. The chlorophyll-a estimation can then be multiplied by a factor of 67

(based on chlorophyll-a being approximately 1.5% of algal biomass) to get estimated algal biomass content of the sample (APHA, 1998).

$$\text{Algal biomass, mg/L} = \text{Chlorophyll-a, mg/L} \times 67 \quad (3.3)$$

The chlorophyll-a determination of biomass is a very innovative procedure for monitoring algae, but suffers from interferences and high variability. The procedures for isolating the biomass and extracting the chlorophyll are limited by laboratory error. In practice, there is no easy way to determine if all the water has been removed and all chlorophyll is extracted, resulting in uncertain results. A major advantage of the method is that only chlorophyll-a is determining biomass, and there are no interferences with wastewater or debris.

3.11 Total Suspended Solids and Volatile Suspended Solids

Solids measurements are methods used in water quality assessment to quantify the total number of solids in the sample. Variants of the method we are interested in include total suspended solids (TSS) and volatile suspended solids (VSS). TSS measures the suspended solids in a sample through filtering out the suspended particulates. The accumulated mass on the filter is the total suspended solids in the sample. VSS is a determination of the organic material in solution by exposure to high temperatures (550 ^{o}C) after a standard TSS method (Sawyer et al., 2003). The amount of mass lost after

exposure to high temperature indicates the amount of organic material that was present in the sample. A detailed procedure was provided in Appendix B.

Total suspended solids and volatile suspended solids are very useful measurements for monitoring biomass, especially in non-wastewater experiments. Although the methods are derived from water quality methods, the results become difficult to distinguish between wastewater debris and actual biomass. Additionally the time requirement is large because of long drying times in the laboratory oven and furnace. However, the values obtained yield very useful units of mg/L which are easily comparable and understandable.

3.12 Nutrient Monitoring

Nitrogen and phosphorous are essential plant nutrients, and important to monitor whenever optimizing plant growth. Also, these nutrients are present in high concentrations in wastewater and considered pollutants for water quality standards. Observing the changes in the amount of these nutrients present can provide information about metabolism and nutrient uptake rates as well as decreases in the effluent wastewater. In order to monitor the changes in these primary nutrients, initial and final measurements must be taken for each sample. The Hach spectrophotometer is equipped with built-in methods that allow for simple measurement of many elements, including total nitrogen, ammonia, nitrate, nitrite, and total phosphorous. Each nutrient was monitored with a separate Hach Test N Tube Plus (TNT+) kit, which are pre-assembled laboratory methods conventionally used to measure these nutrients. Each kit includes detailed instructions of the methods required to make the corresponding measurement.

Figure 19. Nitrate TNT+ Kit with reagents.

Although each test has a different specific method, the concept is similar for all of them. A uniform sample must be collected and placed in a reagent tube. The corresponding reagents are added and allowed sufficient time to react. After the reaction the tubes are placed in the spectrophotometer which had pre-installed programs for each of the TNT+ methods. After recognizing the correct method with a barcode on the tube, the spectrophotometer measures the appropriate wavelengths and returns a concentration of the nutrient. Most of these methods only required 15 – 30 minutes to obtain nutrient concentrations.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Growth Conditions and Optimums

Environmental conditions were monitored throughout the experiment to ensure that growth was not influenced by non-experimental variables. The pH and temperature were observed daily and maintained at specific levels.

4.1.1 pH Conditions

The pH was set and maintained at specific ranges according to optimum conditions found in existing literature, shown in Table 9 (Mayo, 1997; Zittelli et al., 1999; Martinez et al., 2000; Olguin, 2003; Griffith et al., 2009). Aside from minor fluctuations, the pH was relatively constant for all treatments throughout the growth period. As shown in Figure 20, the pH levels were maintained fairly close to the optimum conditions. For the Mixed Freshwater and *Tetraselmis* treatments, the strains were pH tolerant, so conditions were maintained around pH 7.5. Normal fluctuations were observed in pH levels due to the CO_2 regulation.

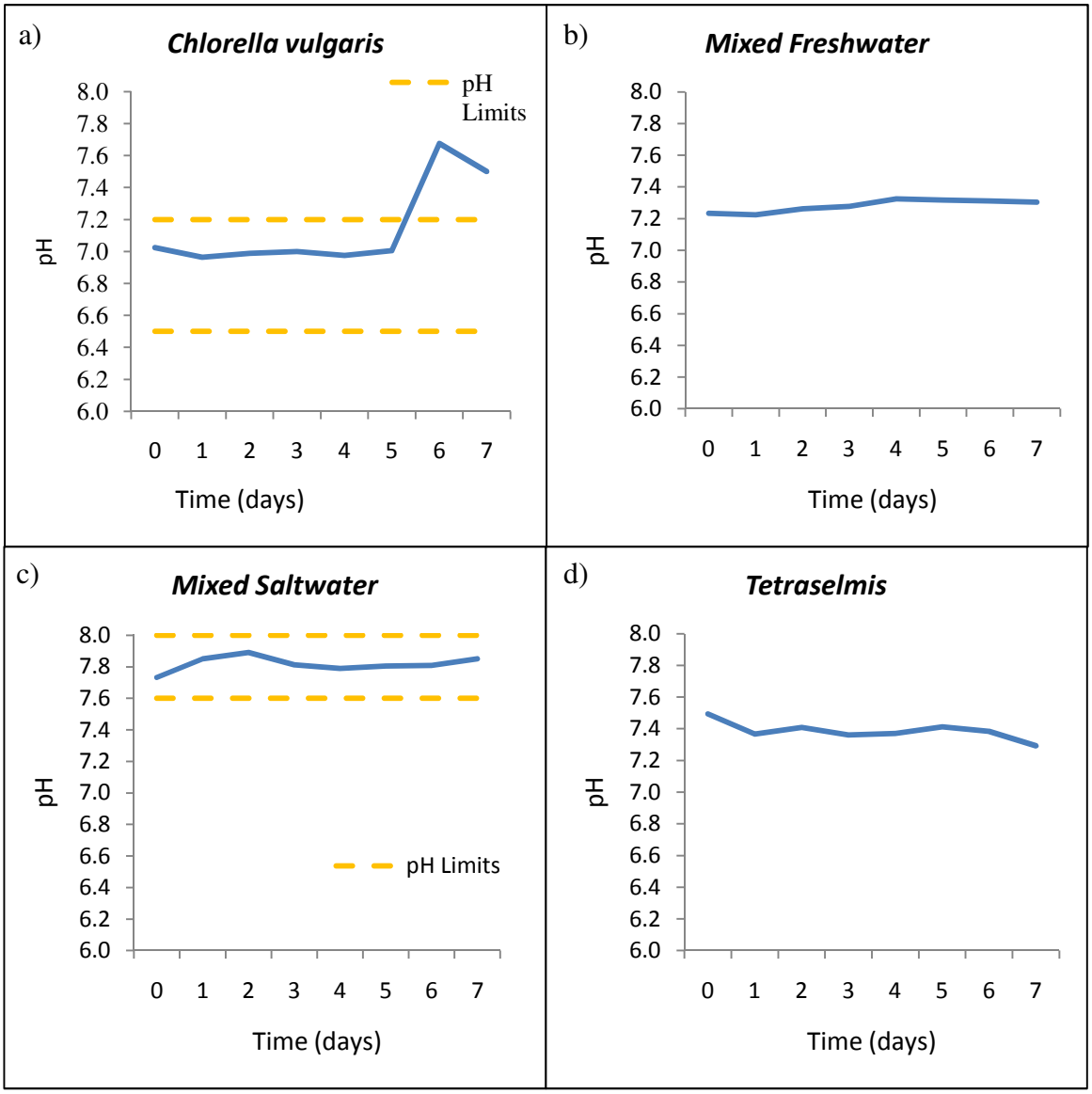


Figure 20. Mean pH conditions and optimums during growth period for a) *Chlorella vulgaris*, b) Mixed Freshwater, c) Mixed Saltwater, and d) *Tetraselmis*.

4.1.2 Temperature Conditions

The temperature was controlled by a heater placed in the photobioreactor that would engage only if the temperature dropped below a set point. This control provided the ability to raise the temperature, but not to decrease it if the photobioreactor became too warm. As shown in Figure 21, most treatment temperatures were able to be

maintained within the desired range, except for the *Tetraselmis* treatment. *Tetraselmis* had an optimum temperature that was below the room temperature, and there were no cooling capabilities on the photobioreactors employed in this study. The temperature optimums were determined based upon existing literature as shown in Table 9.

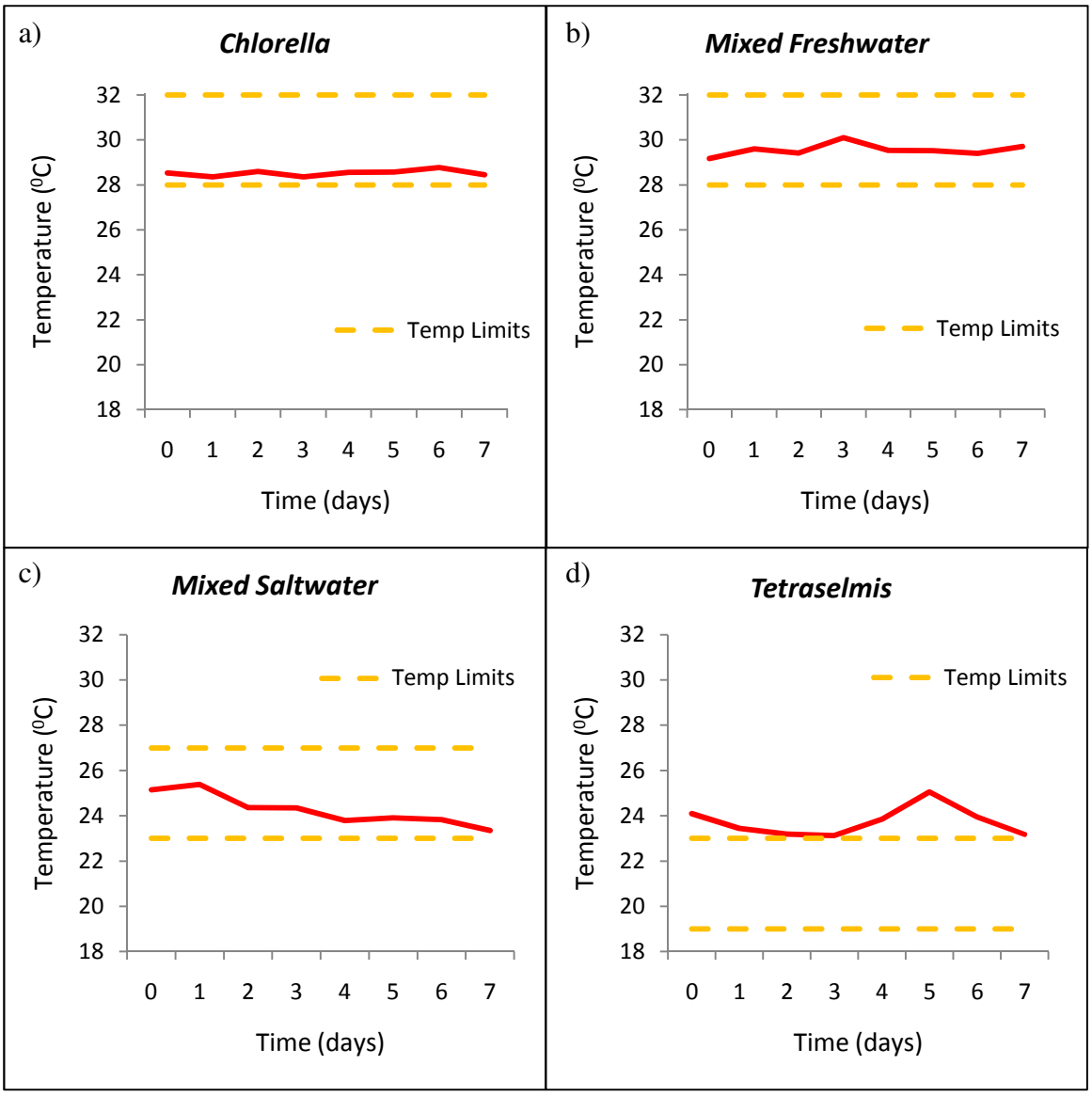


Figure 21. Mean temperature conditions and optimums during growth period for a) *Chlorella vulgaris*, b) Mixed Freshwater, c) Mixed Saltwater, and d) *Tetraselmis sp.*

4.2 Growth Measurements

The results of the experiments are presented in separate sections dedicated to each microalgae quantification method. Although the specific results of each method are different, the cumulative results are summarized in section 4.2.6.

4.2.1 Optical Density via Absorbance

The microalgae growth of each treatment was observed throughout the treatment period of seven days. Although maximum values may occur prior to the final day of the treatment period, it is assumed that the final values are relatively close to the maximum (refer to section 2.6). Figure 22 displays the raw data for the mean absorbance of each strain treatment, at each wastewater concentration. The *Chlorella vulgaris* treatment appears to have higher growth with the *Tetraselmis sp.* and mixed saltwater strains close behind. Rather than one wastewater concentration consistently dominating in all strains, the different concentrations of wastewater exhibited different trends and rankings. However, in all cases, the growth exhibited the expected lag phase and then entered a growth phase thereafter, as was predicted in the literature. The major limitation of the optical density values was that the sample sizes were limited to less than four and usually between one and three replicates.

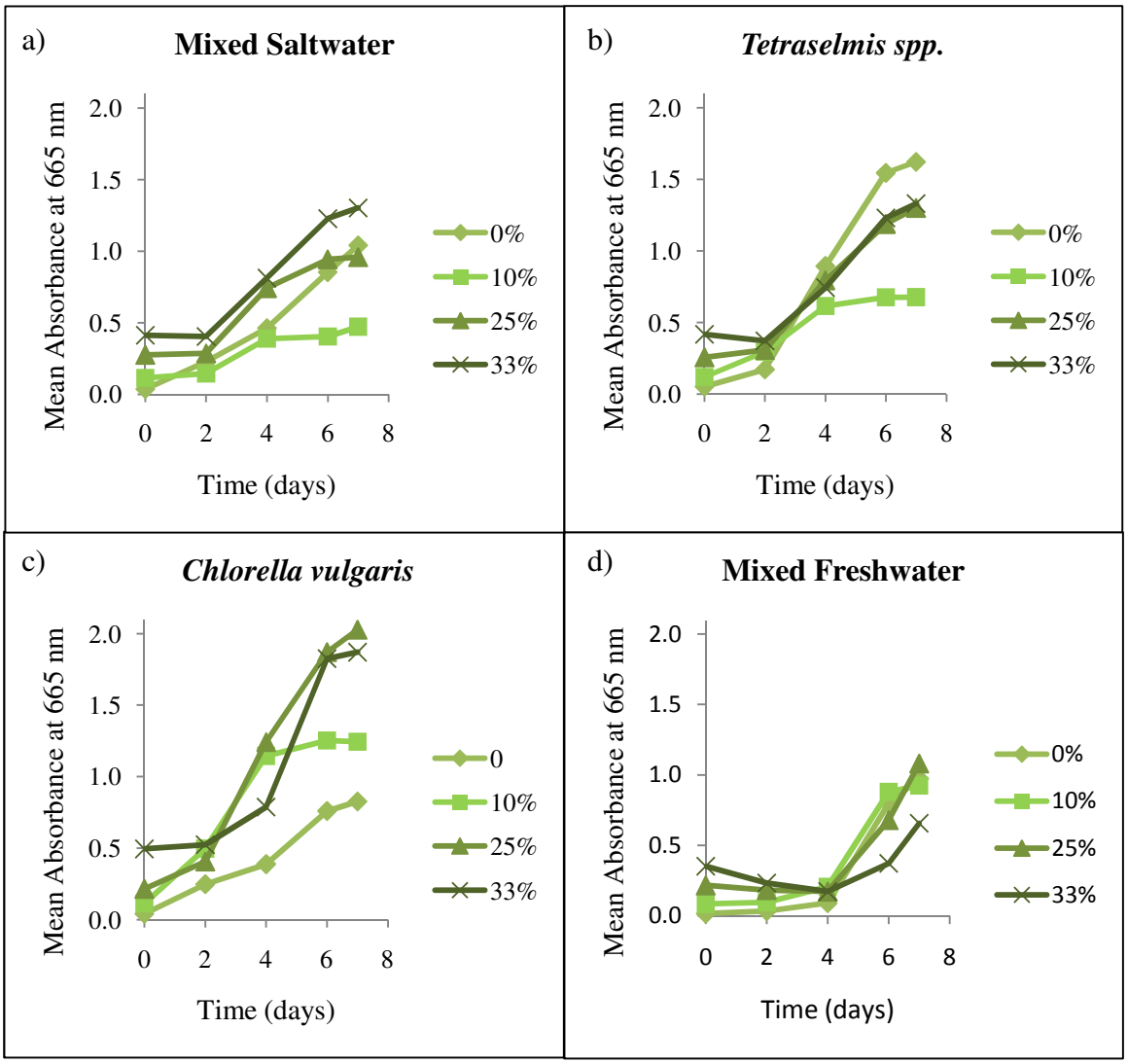


Figure 22. Mean absorbance of different microalgae strain treatments at four different wastewater concentrations.

Figure 23 and Figure 24 demonstrate that there were differences between the different treatments, but do not determine the significance of those differences. The data used in Figure 23 include all wastewater concentrations, plotted by strain, and were simply initial absorbance values subtracted from final absorbance values. Figure 24 includes all strains, plotted by wastewater concentration, and is comprised of initial absorbance values subtracted from final absorbance values. The boxes indicate the

interquartile range of the data, meaning the values between the 25th and 75th percentile. The middle horizontal line bisecting each box represents the median value. The results of Figure 23 and Figure 24 indicate that there are few obvious differences in absorbance for the various treatments and more advanced analysis is needed. The following hypotheses were used to conduct the statistical analysis. The μ symbol refers to the mean with the subscript specifying which treatment group the mean is corresponding to.

Hypothesis testing for strain treatments:

Null hypothesis (H_o): $\mu_C = \mu_{MF} = \mu_{MS} = \mu_T$ Alternative (H_a): at least one differs

Hypothesis testing for wastewater concentration:

Null hypothesis (H_o): $\mu_0 = \mu_{10} = \mu_{25} = \mu_{33}$ Alternative (H_a): at least one differs

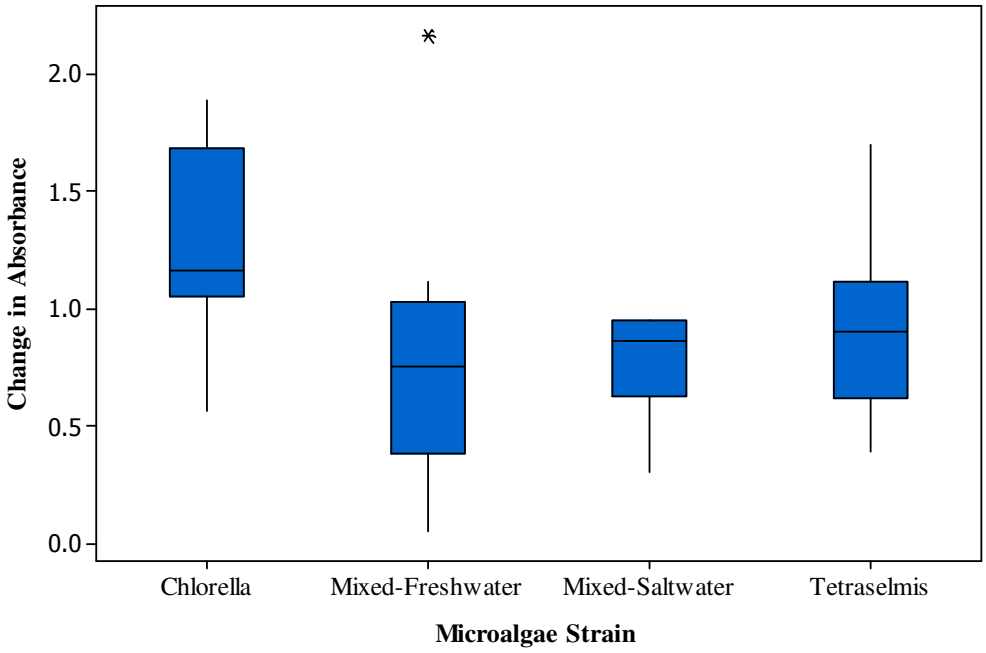


Figure 23. Boxplot of change in absorbance by microalgae strain.

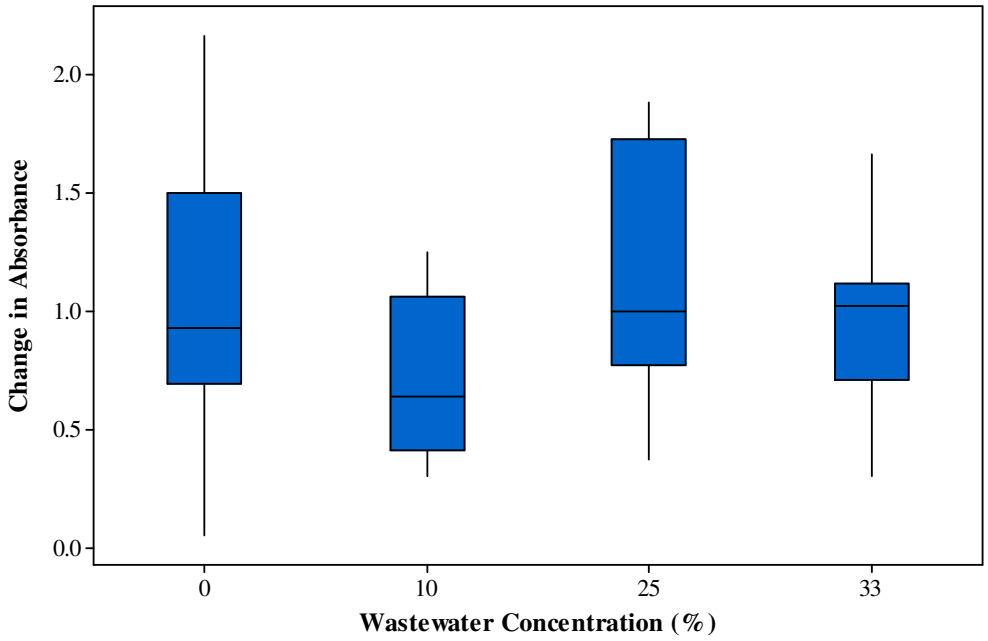


Figure 24. Boxplot of change in absorbance by wastewater concentration.

To conduct the split-plot analysis of variance on the mean change in measured absorbance values, it was necessary to assess the statistical significance of the main effects and interaction effects. The split-plot ANOVA tested the hypotheses mentioned above for the effects of the strain treatment, the wastewater concentration, as well as the interaction between the two. The null hypothesis suggests that there is no statistically significant difference between the population means for the treatments. The alternative hypothesis proposes that at least one of the population means differs from the others. The results of the split-plot ANOVA enabled conclusions to be generated about which hypothesis was to be rejected and helped draw conclusions about the sample data.

Table 10 shows the calculated F-test statistic and corresponding p-value for the main effects and interaction. These statistics were conducted following the methods outlined in Section 3.7. A p-value of less than 0.05 allows us to reject the null hypothesis and conclude that there is a statistically significant difference between at least one of the treatments. Table 11 provides a detailed breakdown of the mean absorbance values and corresponding standard errors of each treatment. The letters adjacent to the sample means are Tukey comparisons; indicating which treatments are not significantly different from one another. For example, each strain treatment has an "a" next to it, which indicates that there is no significant difference between those means. This is not surprising considering the p-value for the strain effect is 0.5135, thereby not allowing the null hypothesis to be rejected. For wastewater concentrations the "a" also means that there is no statistically significant difference between the 0%, 10%, 25%, and 33% treatments. However the significant interaction mandates further analysis of the treatments, which is conducted in Table 12.

Table 10. Test of fixed effects for split-plot ANOVA for optical density measurements.

Effect	F-Test Statistic	p-value
Strain	0.98	0.3909
Wastewater Concentration	3.18	0.0529
Strain*Wastewater Concentration interaction	3.67	0.0024

Table 11. Tukey comparisons for main effects for mean change in absorbance.

Strain			
	Mean		Standard Error
Mixed Fresh	0.74	a	0.1626
Mixed Salt	0.75	a	0.1905
Tetraselmis	1.03	a	0.1132
Chlorella	1.28	a	0.1242

p-value = 0.3909

Wastewater Concentration			
	Mean		Standard Error
10%	0.73	a	0.1241
33%	0.86	a	0.1071
0%	1.09	a	0.1027
25%	1.11	a	0.1058

p-value = 0.0529

Figure 25 provides a visual endorsement of the significance of the interaction as seen by the crossing of the lines for the strain treatments. Figure 25 is simply an alternative to the boxplots in Figure 23 and Figure 24, which also visualize the mean change in absorbance. However, this interaction plot offers a visual depiction of the change in mean absorbance as compared by all of the strain treatments at each wastewater concentration. Additionally, Figure 25 can be used to supplement the Tukey comparisons in Table 11 and Table 12 to help identify the significant differences. Based on the visual comparison provided by the interaction plot, *Chlorella vulgaris* appeared to have the highest mean absorbance for each wastewater concentration while *Tetraselmis sp.* appeared to have the highest mean absorbance for the control group. The mixed freshwater and mixed saltwater treatments alternated having the lowest mean absorbance for the various wastewater concentrations. However, these comparisons are blurred when

looking at Table 12 and it is evident that only a few differences are statistically significant.

To determine the actual statistical significance of the visual differences, Table 12 provides the means and corresponding Tukey comparisons. The *Chlorella* 25% wastewater treatment had the highest mean absorbance change of 1.83. That treatment is only significantly greater than the *Tetraselmis* 33%, *Chlorella* control, and the mixed freshwater 33%, as shown by the absence of the letter "c" in the Tukey comparisons in Table 12. Although there are treatments with lower mean absorbance change than the aforementioned that share the "c", the small and unequal sample size for the optical density measurements is prohibitive of drawing significant conclusions about some inter-strain comparisons. The *Chlorella* 25% treatment is closely followed by the *Tetraselmis* control and the *Chlorella* 33% wastewater treatment with mean absorbance changes of 1.66 and 1.34 respectively. The *Tetraselmis* control is significantly greater than all of the other *Tetraselmis* treatments, as well as the mixed freshwater 33%.

All other comparisons in this table are not detectable with the relatively small sample sizes and corresponding degrees of freedom. Notice that the most of the significant differences that were detected were comparing treatments within the same strain, which have identical sample size. These results do not enable many conclusions to be drawn about the comparisons between the treatment groups using the optical density biomass estimation method.

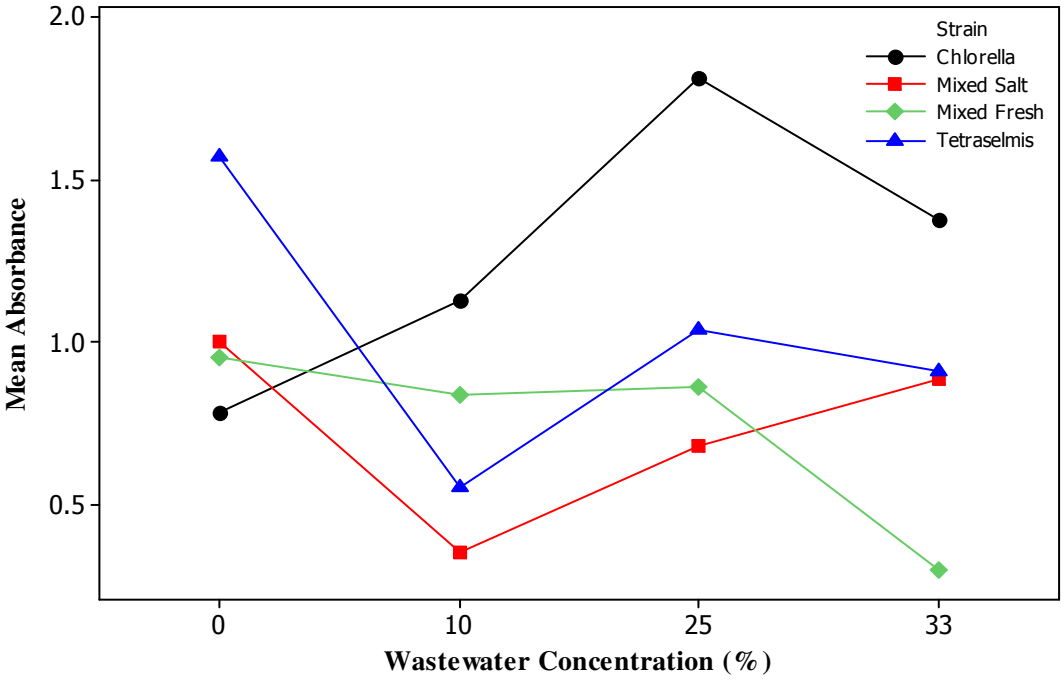


Figure 25. Interaction plot for mean change in absorbance values for each treatment.

Table 12. Tukey comparisons for interaction effects for mean change in absorbance.

Strain/Wastewater Concentration Interaction			
	Mean		**Standard Error**
Mixed Fresh 33%	0.30	a	0.3242
Mixed Salt 10%	0.46	a b c	0.1752
Tetraselmis 10%	0.51		0.1209
Mixed Salt 25%	0.72	a b c	0.1752
Chlorella 0%	0.80	a b	0.1633
Mixed Fresh 10%	0.84	a b c	0.4178
Mixed Salt 33%	0.85	a b c	0.1937
Mixed Fresh 25%	0.86	a b c	0.3242
Tetraselmis 33%	0.91	c	0.1193
Mixed Salt 0%	0.95	a b c	0.1937
Mixed Fresh 0%	0.96	a b c	0.2939
Tetraselmis 25%	1.04	a c	0.1287
Chlorella 10%	1.12	b c	0.1633
Chlorella 33%	1.36	b c	0.1633
Tetraselmis 0%	1.67	b c	0.1343
Chlorella 25%	1.83	c	0.1633

The Tukey comparisons in Table 11 did statistically support the literature in concluding that wastewater media could effectively support the microalgae growth. The wastewater treatments were statistically indistinguishable from the control treatment of plant food. This conclusion suggests that wastewater could supplant the need for plant food in growing microalgae. The trends of the mean change in absorbance shown in Figure 23 follows a pattern suggested by existing literature that increasing wastewater concentration is beneficial until it reaches a concentration where growth becomes limited. For the optical density portion of the microalgae growth analysis, it appears that the concentration that yields maximum microalgae biomass is approximately 25% wastewater. The strain effects did not permit concluding that one strain treatment performed significantly better than another. This is interesting because some wastewater treatment studies suggest a mixed culture would outperform a pure culture in the uncertain conditions of wastewater media. The evidence of this study does not confirm that assessment.

4.2.2 Cell Counts

The method for determining growth by mean cell counts was calculated by subtracting initial cell counts from final cell counts. The cell count values were recorded with both the desired microalgae strain being counted as well as any unidentified strains that were present. In order to see the true representation of algal biomass present from the cell counts, both desired microalgae cells and unidentified cells must be totaled. Figure 26 shows the mean cell counts and unidentified cells for each strain and each wastewater concentration. The error bars were based upon the standard error of the mean for the microalgae cell counts.

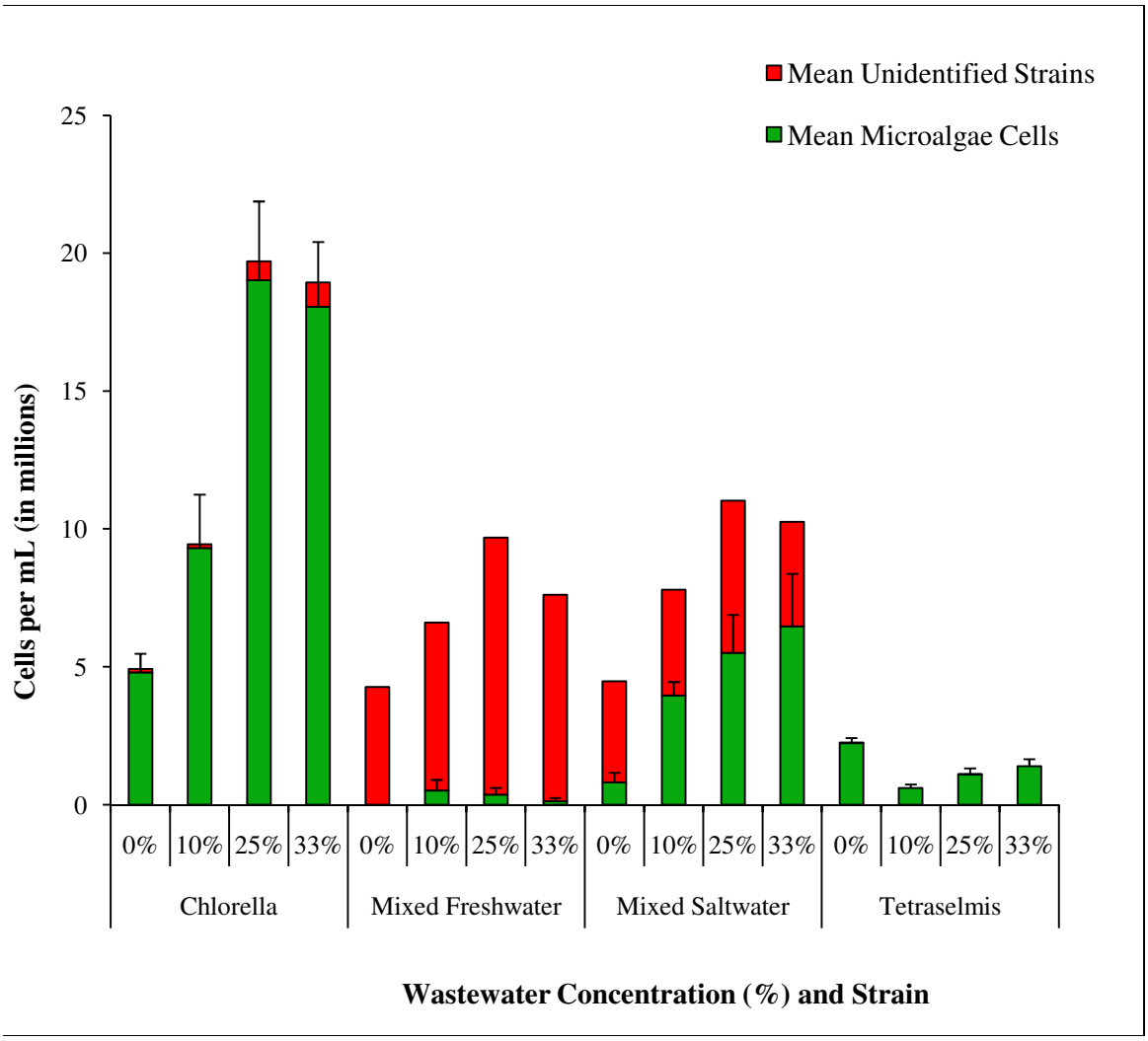


Figure 26. Mean microalgae and unidentified cell count for treatment.

Figure 26 suggests *Chlorella vulgaris* exhibited larger cell counts than the other strains with maximum values at approximately 20 million cells per milliliter. The large presence of unidentified cells for the mixed culture treatments highlights the high species diversity present in those cultures. The green bars for the mixed cultures indicate the incidence of original microalgae strains *Scendesmus sp.* and *Nanncochloropsis sp.* While *Tetraselmis sp.* seemed to have relatively low cell counts, as compared to the other strains, it also had very low presence of unidentified cells. It is important to consider the

larger cell size of *Tetraselmis sp.* cells (10 – 15 µm) (Nash and Novotny, 1995) as compared to the other microalgae strains like *Chlorella vulgaris* (2 – 12 µm) (Bold and Wynne, 1978). The resulting cell counts for *Tetraselmis sp.* are significantly lower, despite high sample densities indicated in other growth measures. The maximum mean cell counts appeared to occur at the 25% wastewater concentration for all three strain treatments; *Chlorella vulgaris,* mixed freshwater, and mixed saltwater and at the 0% wastewater concentration for *Tetraselmis sp*.

Table 13. Test of fixed effects for split-plot ANOVA for cell counts.

Effect	F-Test Statistic	p-value
Strain	48.40	0.1052
Wastewater Concentration	18.01	0.1711

Table 14. Tukey comparisons for main effects for mean change in cell count.

Strain			
	Mean		Standard Error
Tetraselmis	1,339,958	a	704,494
Mixed Fresh	7,042,708	a	704,494
Mixed Salt	8,390,667	a	704,494
Chlorella	13,254,167	a	704,494

p-value = 0.1052

Wastewater Concentration			
	Mean		Standard Error
0%	3,980,875	a	704,494
10%	6,114,292	a	704,494
33%	9,550,375	a	704,494
25%	10,381,958	a	704,494

p-value = 0.1711

The split-plot ANOVA shown in Table 13 conducted on the cell counts resulted in an inability to conclude that any of the main effects are significantly different. This is likely attributable to the very large standard error associated with the mean cell count values for each treatment. The large standard errors indicate that the data for cell counts had high variability and/or low levels of replication, and makes statistically significant

conclusions more challenging to generate. Despite the noticeably different means associated with each treatment, shown in Table 14, the statistics do not suggest any significant difference. Table 13 illustrates that the p-values associated with the two main effects are too large to reject the null hypothesis and conclude that there is any significant difference between the treatment means for cell counts. Any further conclusions about differences in the cell count data would not be statistically significant.

4.2.3 Cell Divisions per Day

Cell counting throughout the growth period provided a unique opportunity to calculate rates of cell division, or microalgae growth. The division rates for most treatments exhibited declining rates of division through the course of the growth period. Table 15 displays the calculated cell divisions per day for each microalgae strain treatment at intervals of the growth period. All strain treatments exhibited a similar pattern of decreasing division rates toward the end of the growth period. When considering the sigmoidal curve for microbial growth kinetics, as mentioned in section 2.6, this pattern makes sense. The microalgae are likely starting in the exponential growth phase and slowing into the stationary phase. The maximum divisions per day occurred in *Chlorella vulgaris* treatments in the first two days of growth. Because cell counts were not taken daily for the treatment period, these division rates were averages over a two-day period. These rate-based population measures are essential to maximizing biomass yield in commercial scale applications.

Table 15. Cell divisions per day for each treatment.

	Divisions per Day			
Days of Growth Period	**0 to 2**	**2 to 4**	**4 to 6**	**6 to 7**
Chlorella	1.72	0.87	0.29	0.02
Mixed Freshwater	1.43	1.15	1.05	0.21
Mixed Saltwater	1.09	0.88	0.24	0.14
Tetraselmis	0.69	1.27	0.12	0.13

4.2.4 Algal Biomass by Chlorophyll-a Determination

Chlorophyll-a was measured every other day as well as at initial and final readings. The total growth was estimated by subtracting the initial from the final values. The algal biomass measurements by chlorophyll-a determination for all strain treatments showed similar behavior with regards to the effect of wastewater concentrations. The control group (0% wastewater) had moderate growth, with the other groups increasing with increasing wastewater concentration. Figure 27 illustrates that growth for each treatment was fairly consistent except for the *Tetraselmis* control group having very high growth. Aside from the aforementioned instance, the 33% wastewater treatments appeared to experience the greatest growth. Because of the nature of this chlorophyll-a method, it is impossible to determine what percentages of the measured values were attributable to contamination.

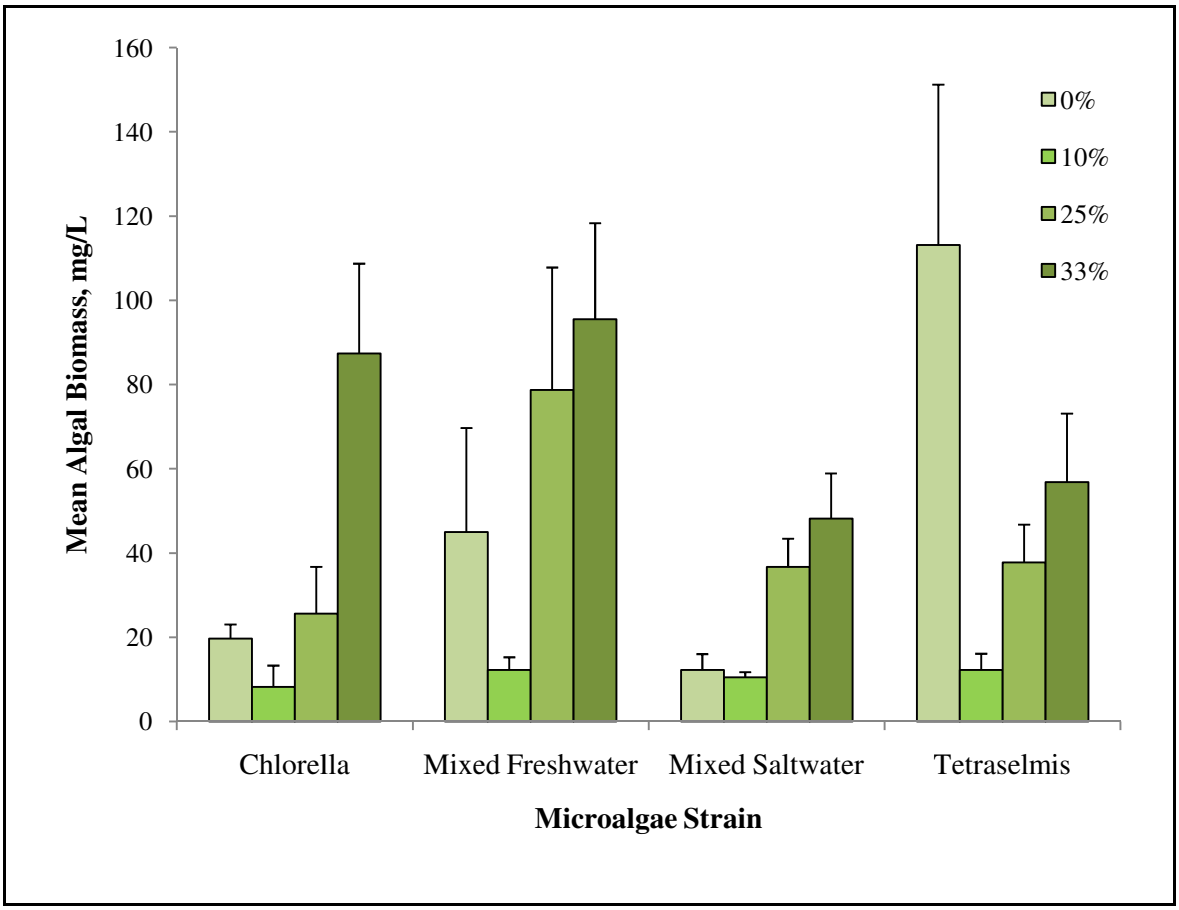


Figure 27. Mean algal biomass by chlorophyll-a determination for different microalgae strains at four different wastewater concentrations.

Table 16. Test of fixed effects for split-plot ANOVA for algal biomass.

Effect	F-Test Statistic	p-value
Strain	0.39	0.4552
Wastewater Concentration	8.29	<0.0001
Strain*Wastewater Concentration interaction	1.70	0.1294

Table 17. Tukey comparisons for main effects for mean change in algal biomass.

Strain			
	Mean		**Standard Error**
Chlorella	35.22	a	11.51
Mixed Salt	41.30	a	11.51
Tetraselmis	54.96	a	11.51
Mixed Fresh	57.87	a	11.51

p-value = 0.4552

Wastewater Concentration				
	Mean			**Standard Error**
10%	10.81	a		2.38
25%	44.51		b	7.47
0%	61.89		b	18.28
33%	72.15		b	8.99

p-value = <0.0001

The split-plot ANOVA for the mean algal biomass supports the data shown in Figure 27 by concluding that there is a statistically significant effect of wastewater concentration but not a significant effect for the strain treatment. This conclusion is exemplified in Table 16 by the p-value for strain being 0.4552, prohibiting rejection of the null hypothesis. Alternatively, the p-value for wastewater concentration in Table 16 is less than 0.0001, allowing for rejection of the null hypothesis. The corresponding Tukey comparison tables are shown in Table 17 for a further breakdown of the analysis. For wastewater concentration, the letters indicate some significant differences between the treatments as seen in Table 17. The lowest mean algal biomass, the 10% wastewater concentration, with a mean algal biomass of 10.81 mg/L, is significantly different from all of the other treatments. The remaining treatments are not significantly different from one another. The highest mean algal biomass treatment, the 33% wastewater, has a mean algal biomass of 72.15 mg/L.

Similar to the results from the optical density method, the control treatment was not distinguishable from at least one of the wastewater concentrations. For the algal biomass method, the 33% wastewater treatment was the highest growth, although statistically indistinguishable from the control. Again, these results suggest that plant

food could effectively be replaced with wastewater as growth media. Another consistency with the optical density method analysis is that strain effect was not statistically significant, thereby giving no advantage to one particular strain treatment.

4.2.5 Total Suspended Solids and Volatile Suspended Solids

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured simultaneously throughout the course of the experiments. VSS was the desired variable because the values are focused on organic components in the sample and because biomass is organic, the VSS measurements should provide more accurate representations of the biomass in the samples than TSS. Figure 28 provides a visual breakdown of the change in composition of the solids present in the samples throughout a growth period to help illustrate that concept. While both TSS and VSS increased, VSS became the more predominant constituent as time progressed and biomass increased. Additionally, Table 18 provides the complete values for mean TSS and VSS at both initial and final values. Although these values were the combined total for all strains, they were broken down by wastewater concentration to illustrate the effects of wastewater on the change in solids content. As expected the initial values for TSS and VSS increase with increasing concentration of wastewater. However, for the final readings, the disparities are less clear; probably due to the effects of biomass in the samples. It is important to note that severe outliers were eliminated from the TSS/VSS dataset because some early methods were conducted incorrectly and the data was irrelevant. Based on the previous information, for the analysis of the growth and nutrient data, VSS will be used instead of TSS.

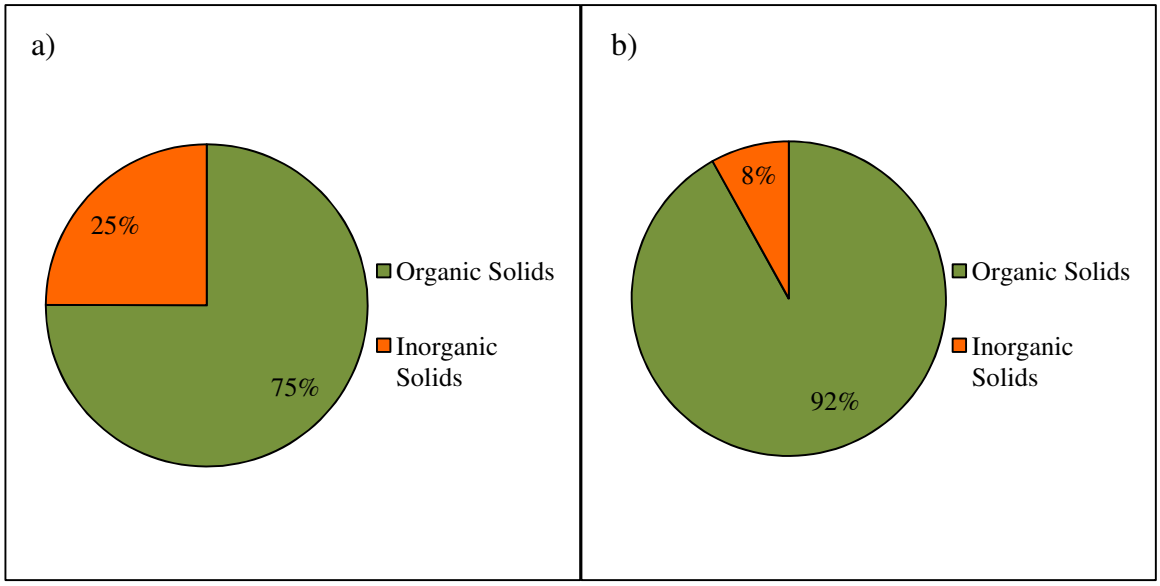


Figure 28. Suspended solids for a) initial and b) final readings at 33% wastewater.

Table 18. Mean initial and final TSS and VSS values by wastewater concentration.

Mean TSS and VSS, mg/ L

Wastewater Concentration (%)	VSS Initial	TSS initial	VSS Final	TSS Final	Volatile percentage of TSS
0	17.1	50.2	418.5	479.0	87%
10	75.3	110.5	413.7	431.2	96%
25	103.5	144.4	622.3	798.0	78%
33	193.4	257.7	663.3	721.4	92%

The mean measured values for VSS (in mg/L) were significantly greater than those of the values measured for algal biomass via the chlorophyll-a method. This inconsistency can potentially be due to wastewater interferences such as organic debris or bacteria that inflate the measured VSS values beyond the true values of algal biomass. Figure 29 shows the mean growth of VSS (VSS initial subtracted from VSS final) for each treatment group. The values are fairly consistent with averages ranging from 200

mg/L to 1,000 mg/L. Although larger than the algal biomass values, the trends are similar with means increasing with wastewater concentration and *Tetraselmis sp.* having larger numbers and a spike for that control treatment.

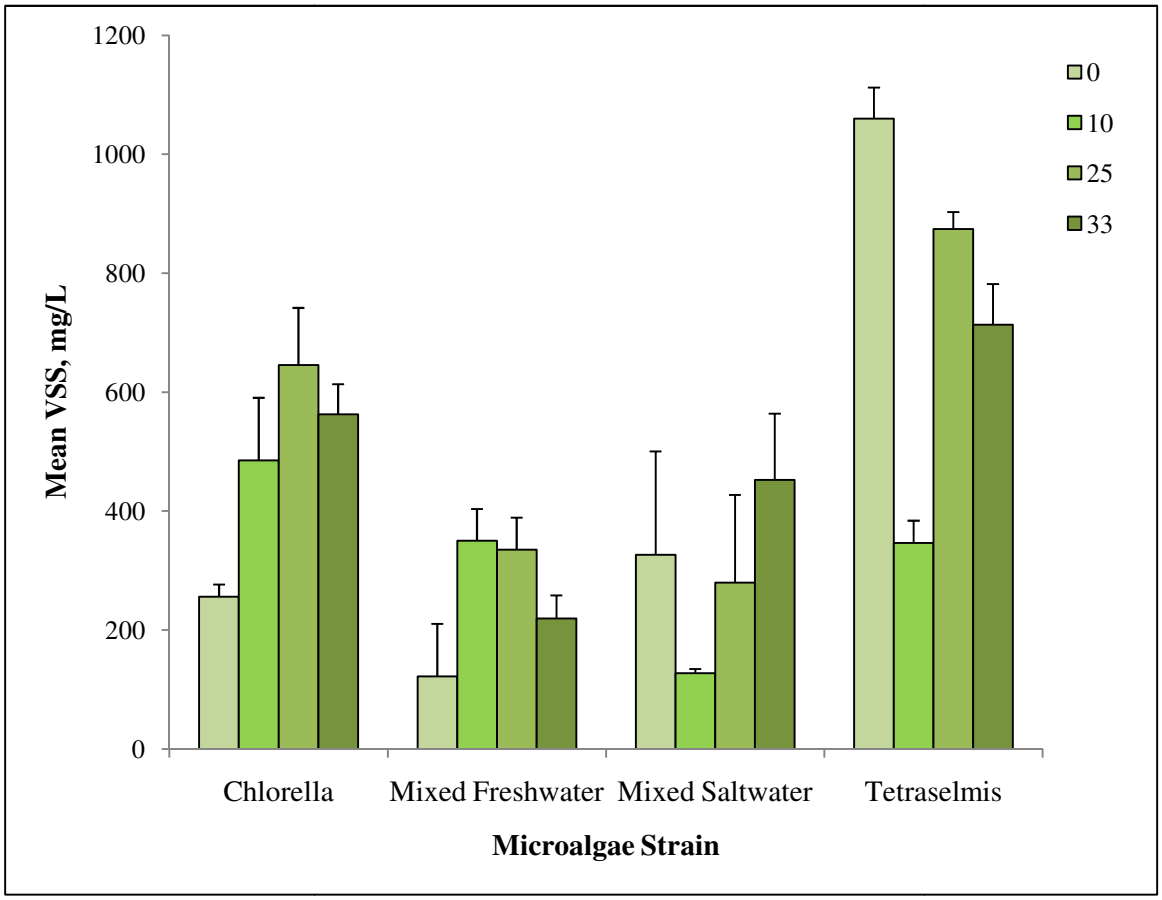


Figure 29. Mean volatile suspended solids for different microalgae strains at four different wastewater concentrations.

Table 19. Test of fixed effects for split-plot ANOVA for volatile suspended solids.

Effect	F-Test Statistic	p-value
Strain	6.76	0.0124
Wastewater Concentration	5.56	0.0021
Strain*Wastewater Concentration interaction	6.51	<0.0001

Table 20. Tukey comparisons for main effects for mean change in volatile suspended solids.

Strain	Mean		Standard Error
Mixed Fresh	256.67	a	76.54
Mixed Salt	305.33	a	88.47
Chlorella	486.12	a b	88.46
Tetraselmis	746.13	b	88.47

p-value = 0.0124

Wastewater Concentration	Mean		Standard Error
10%	370.17	a	51.75
0%	401.42	a	52.38
33%	481.98	a b	51.24
25%	540.68	b	51.89

p-value = 0.0021

The split-plot ANOVA conducted on the mean volatile suspended solids values shown in Table 19 yields a statistically significant interaction effect, as well as the strain and wastewater effects independently. Because the low p-values allow for rejecting the null hypothesis, it is evident that there is a significant difference between the population means for at least one treatment. Due to a significant interaction effect, the Tukey comparison in Table 21 was generated to provide a detailed breakdown on each treatment in comparison with one another.

Table 20 illustrates the Tukey comparison for the main effects. For the strain treatment effect, the mixed freshwater, mixed saltwater, and *Chlorella vulgaris* treatments were not significantly different from one another. The *Tetraselmis sp.* treatment with the greatest mean VSS growth of 746 mg/L did differ significantly from the two groups with the lowest mean VSS (the mixed freshwater and mixed saltwater treatments), but was not significantly different from the *Chlorella vulgaris* treatment. The Tukey comparison on wastewater concentration effect determined that the two treatments with the lowest mean VSS, 0% and 10%, were not significantly different from one another or the 33% treatment. The middle value, the 33% treatment with mean VSS growth of 482 mg/L, was not significantly different from any of the other treatments.

The treatment with the greatest mean VSS of 541 mg/L, the 25% wastewater concentration, was significantly different from 0% and 10%, but not the 33% treatment.

The split-plot ANOVA provides interesting interpretation of the data shown in Figure 29, with relatively high variance detection ability. While the data shown in Figure 29 for VSS seem similar to the data for algal biomass, shown in Figure 27, the major difference is the lower standard errors for the VSS data. The lower standard error (more precise data) for VSS allows for detecting more statistically significant differences.

Figure 30 is an interaction plot showing mean change in VSS by wastewater concentration for each strain treatment. This figure points out the trends for each strain and assists in analyzing the Tukey comparisons generated in Table 20 and Table 21. The *Tetraselmis sp.* value for the control (0% wastewater) is clearly higher than any other treatment with a mean growth of 1021 mg/L, and this is supported by it sharing letters "e" with only one other treatment in the Tukey comparison table. This Tukey comparison concludes that the *Tetraselmis sp.* control has significantly higher VSS change than most of the other treatments. Although the *Tetraselmis sp.* 25% treatment has a mean VSS growth of 864 mg/L, the difference is not statistically significant from the control treatment. Based on these results, it can be suggested that *Tetraselmis sp.* can be grown effectively with 25% wastewater as a substitute for the use of traditional plant food. The *Tetraselmis sp.* 33% wastewater treatment had a similarly high VSS growth of 722 mg/L, but was not significantly greater than most other treatments. Similarly, the *Chlorella vulgaris* 25% wastewater treatment had moderately large VSS change with a mean growth of approximately 640 mg/L but was not significantly greater than most

other treatments. Based on the Tukey comparisons, only the *Tetraselmis* 0% and *Tetraselmis* 25% are significantly greater than the majority of other treatments.

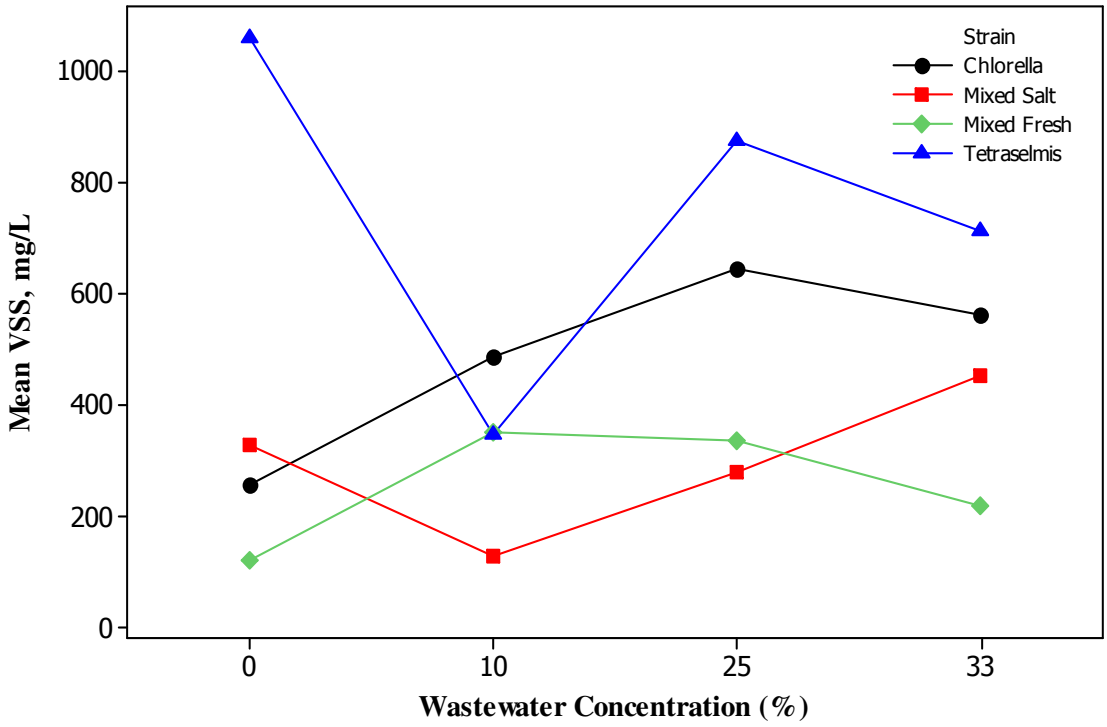


Figure 30. Interaction plot for mean volatile suspended solids for each treatment.

The results of the split-plot ANOVA of the volatile suspended solids data are consistent with the literature which suggested that microalgae could be grown in various wastewater media. However, in comparing the wastewater treatments to a control group of plant food, the results of this study demonstrate competitive, if not equivalent, microalgae growth in wastewater. Table 20 even illustrates that the 25% wastewater treatment exhibited statistically significantly greater mean VSS growth as compared to the control treatment. When looking at Figure 28, the control treatments for all strains,

excluding *Tetraselmis sp.,* had lower VSS growth than at least one of their corresponding wastewater treatments.

Table 21. Tukey comparisons for interaction effects for mean change in volatile suspended solids.

Strain/Wastewater Concentration Interaction			
Treatment	**Mean**		**Standard Error**
Mixed Fresh 0%	138.76	a	92.07
Mixed Fresh 33%	216.81	a b	92.07
Mixed Salt 0%	219.99	a b c	105.59
Chlorella 0%	225.49	a c	109.00
Mixed Salt 10%	260.76	a b c	111.33
Mixed Salt 25%	320.88	a b c d	109.03
Mixed Fresh 10%	333.24	a b c d	92.07
Mixed Fresh 25%	337.86	a b c d	92.07
Tetraselmis 10%	377.45	a b	105.59
Mixed Salt 33%	419.70	a b c d	104.04
Chlorella 10%	509.20	a b c d e	104.02
Chlorella 33%	569.87	a b c d e	109.00
Chlorella 25%	639.91	b c d e	104.02
Tetraselmis 33%	721.53	c d e	104.04
Tetraselmis 25%	864.08	d e	109.03
Tetraselmis 0%	1021.44	e	111.33

Unlike any of the other method analyses, the VSS results indicated a significant difference between the strain treatments. Interestingly, the significant difference demonstrated that *Chlorella vulgaris* and *Tetraselmis sp.* had the highest mean change in VSS with 486 mg/L and 786 mg/L respectively. However, the *Tetraselmis sp.* treatment was the only treatment that was significantly greater than the other strain treatments. This supports the literature that suggests *Tetraselmis sp.* would grow effectively in wastewater media and has positive implications for pure culture microalgae cultivation in wastewater.

4.2.6 Summary of Growth Analysis

Each method for quantifying microalgae biomass had flaws and was limited in results. These errors were abated by combining the results of all of the methods to infer conclusions about the experimental treatments. As shown Table 22, the four methods used each yielded slightly different results. However, the recurring prevalence of the *Tetraselmis* 0% and *Chlorella* 25% indicate that those treatments repeatedly outperformed the other treatments. As outlined by the volatile suspended solids split-plot ANOVA, the *Tetraselmis* and *Chlorella* strains dominated the highest biomass results. With regard to comparing wastewater concentration alone, the chlorophyll-a sections illustrated that 33% and 25% wastewater were statistically indistinguishable from the control group. Unfortunately the variability in the cell count data did not allow for statistically significant results to be established.

Ultimately the 25% and 33% wastewater concentrations seemed to effectively increase biomass during these experiments. Also, the pure cultures *Chlorella vulgaris* and *Tetraselmis sp.* grew effectively in the wastewater media. Although the results are convoluted by the multiple methods and high variability, it is safe to make such generalizations. For more conclusive results another study could be conducted with narrower research parameters and higher replication to achieve more clear comparisons.

Table 22. Summary table for microalgae growth by method

	Optical Density	Cell Count	Chlorophyll-a	Volatile Suspended Solids
Top Ranking Treatments	1. *Chlorella* 25% 2. *Tetraselmis* 0%	*Chlorella* 25% appeared to be highest	1. 33% 2. 0% 3. 25%	1. *Tetraselmis* 0% 2. *Tetraslemis* 25% 3. *Tetraselmis* 33% 4. *Chlorella* 25%
Comments	Lacking statistical significance due to variability and small sample size	No significant effects	No significance for strain and only 10% WW was significantly less	Significant effects suggest 25% and 33% and *Tetraselmis* and *Chlorella* highest respectively

4.3 Nutrient Observation, Uptake, and Analysis

4.3.1 Nutrient Uptake

The nutrients observed for this experiment were total nitrogen, ammonium, nitrate, nitrite, and total phosphorus. The values were measured as the initial and final observations, with the nutrient removals calculated from those values. Table 23 provides a breakdown of the initial nutrient concentrations available in each treatment. The significance of the initial nutrient values is to illustrate that there was substantially more of some nutrients, such as ammonium and nitrate, as compared to nitrite. Also, the values in Table 23 indicate the initial growing conditions for nutrition available in each treatment group. The initial nutrient concentrations were also used as predictors for microalgae growth in the multiple regression analysis.

Table 23. Mean initial nutrient concentrations.

	Wastewater Concentration (%)	Total N (mg/L)	Ammonium (mg/L)	Nitrate (mg/L)	Nitrite (mg/L)	Total P (mg/L)
Chlorella vulgaris	0	58.60*	18.90*	2.49	0.02	14.00
	10	15.43	0.93	8.75	0.11	10.44
	25	37.37	1.18	22.30	0.23	11.68
	33	46.70	1.51	26.63	0.41	12.74
Mixed Freshwater	0	31.20*	46.40*	2.60	0.31	12.11
	10	19.28	1.93	9.77	0.48	10.38
	25	39.53	1.96	22.12	0.59	11.68
	33	48.03	2.43	26.58	0.44	12.18
Mixed Saltwater	0	23.14	18.18	0.72	0.06	6.70
	10	2.15	1.15	2.28	0.08	2.15
	25	9.93	2.24	5.55	0.19	3.80
	33	15.62	1.77	7.81	0.55	8.28
Tetraselmis sp.	0	8.26	10.35	0.53	0.02	3.54
	10	3.13	1.03	2.19	0.09	2.02
	25	8.65	3.56	5.79	0.27	3.94
	33	16.63	2.44	7.93	0.30	8.73

* values were based upon ≤ 2 replicates

The changes in nutrient concentration were determined by comparing final versus initial nutrient concentrations. Table 24 provides the percentage change in nutrients in each sample, broken down by strain treatment and wastewater concentration. Initial assessment of Table 24 suggests that total nitrogen experienced inconsistent changes during the growth period. Ammonium exhibited some moderate decreases, but had larger increases in the higher concentrations of wastewater. Nitrate showed strong reductions with very low variability, aside from the control groups which were inconsistent. Nitrite demonstrated very inconsistent change but generally seemed to increase substantially in almost all control treatments. Total phosphorus also exhibited inconsistent change

although it appears that the freshwater treatments had modest reductions while the saltwater treatments had significant increases. The very high reductions in nitrate are consistent with the expected nutrient uptake as stated in the literature. However, some experiments suggested close to 100% removal for total nitrogen, ammonium, nitrate, and phosphorus. These findings do not seem to agree which may be a result of a shorter growth period in this experiment, or experimental error.

Table 24. Percentage change in nutrient concentration.

	Wastewater Concentration (%)	Total N	Ammonium	Nitrate	Nitrite	Total P
Chlorella vulgaris	0	22%*	-100%*	-12%	990%	2%
	10	-9%	-1%	-85%	273%	-7%
	25	-56%	40%	-87%	174%	-8%
	33	-46%	21%	-87%	78%	-11%
Mixed Freshwater	0	-100%*	-100%*	-24%	1130%	-22%
	10	-22%	-13%	-88%	69%	-10%
	25	-29%	64%	-89%	55%	-16%
	33	-38%	55%	-79%	204%	-13%
Mixed Saltwater	0	-18%	-67%	193%	1530%	76%
	10	112%	-31%	-62%	122%	-8%
	25	31%	53%	-59%	164%	172%
	33	-27%	70%	-59%	65%	57%
Tetraselmis sp.	0	2120%	65%	974%	2155%	211%
	10	-49%	122%	-52%	143%	392%
	25	-18%	103%	-51%	103%	147%
	33	-51%	185%	-55%	95%	40%

*values were based upon ≤2 replicates.

Table 25. 95% confidence intervals on mean nutrient changes.

	Wastewater Concentration (%)	Total N 95% CI	Ammonium 95% CI	Nitrate 95% CI	Nitrite 95% CI	Total P 95% CI
Chlorella vulgaris	0	**10% , 34%**	-100% , -100%	-33% , 10%	**702% , 1279%**	-1% , 5%
	10	-18% , 0%	-19% , 18%	**-88% , -82%**	**175% , 372%**	-17% , 3%
	25	**-84% , -27%**	**14% , 66%**	**-88% , -86%**	**119% , 230%**	**-14% , -1%**
	33	**-78% , -14%**	-29% , 71%	**-89% , -85%**	**13% , 140%**	-24% , 2%
Mixed Freshwater	0	-68% , 31%	**-96% , -38%**	**-**16% , 401%	-425% , 3486%	**22% , 129%**
	10	-143% , 367%	-61% , 0%	**-69% , -54%**	**104% , 141%**	-22% , 5%
	25	-117% , 178%	-42% , 148%	**-67% , -51%**	**47% , 281%**	**159% , 185%**
	33	-54% , 1%	-9% , 148%	**-68% , -51%**	**12% , 118%**	-4% , 117%
Mixed Saltwater	0	-100% , -100%	-100% , -100%	-57% , 8%	**251% , 2009%**	-53% , 9%
	10	-43% , 0%	-94% , 68%	**-90% , -85%**	-72% , 210%	**-13% , -7%**
	25	**-41% , -17%**	-63% , 191%	**-90% , -88%**	-44% , 153%	**-23% , -9%**
	33	**-47% , -28%**	-79% , 188%	**-99% , -58%**	-91% , 499%	**-14% , -12%**
Tetraselmis sp.	0	-987% , 5229%	-127% , 258%	**381% , 1566%**	**1440% , 2871%**	**96% , 325%**
	10	**-80% , -17%**	**12% , 233%**	**-60% , -44%**	**64% , 222%**	**109% , 674%**
	25	-57% , 22%	-27% , 233%	**-60% , -41%**	-4% , 211%	**55% , 239%**
	33	**-69% , -32%**	**43% , 327%**	**-61% , -49%**	**34% , 156%**	-28% , 108%

The confidence intervals displayed in Table 25 illustrate the high variability in much of the nutrient data, as shown by the large ranges. In the table, the first number is the lower confidence limit and the second number is the upper confidence limits. The most meaningful results derived from these confidence intervals are depicted by the treatments highlighted in bold in Table 25. These bold treatments do not include zero in the 95% confidence interval, thereby suggesting that there was a statistically significant change in the nutrient concentration. For example, the interpretation of the *Chlorella* 10% wastewater treatment for nitrate states that we are 95% confident that the nitrate reduction would be between 82% and 88% for all *Chlorella vulgaris* cells in 10% wastewater. This result is very important to drawing the statistically significant conclusion that algae cells will reduce nitrate concentrations under these treatment conditions. Alternatively, the non-bold treatments in Table 25 indicate confidence intervals that include zero. These treatments do not allow us to conclude that there was significant change in the nutrient levels.

The results from Table 25 for most of the nutrients, excluding nitrate, offer far more difficult to interpret conclusions. For total nitrogen, most of the significant confidence intervals are negative, suggesting a tendency toward declining total nitrogen during the growth period. This partially agrees with the literature in that nitrogen levels decreased, although not as substantially as expected. Very few ammonium treatments yielded significant confidence intervals, although the significant treatments were usually corresponding to increasing concentrations. Literature suggested much greater ammonium decreases, especially considering the expected affinity the microalgae cells would have for ammonium over nitrate. However, these results displayed much greater

nitrate reductions than ammonium. The nitrite confidence intervals were predominantly increasing concentrations, sometimes by very large orders of magnitude. Total phosphorus exhibited mixed results with both positively and negatively significant confidence intervals. With no apparent trends in the treatments for phosphorus, the results contrast the expected decreases suggested by literature.

As outlined in Section 2.5.3, ammonium and nitrate have the greatest significance to microalgae cultivation. For this reason, these two nutrients will be analyzed in the greatest depth. Although the nitrate reductions were very consistent, the reductions in ammonium were somewhat inconsistent between the treatment groups. An important note about the ammonium values is their dependency on the initial ammonium present in the sample (in mg/L). In general, the control groups had larger ammonium concentrations as compared to the wastewater treatment groups. Based on the literature, the algae will consume ammonium until it is depleted, then utilize available nitrate. Table 26 verifies this relationship by showing a significant negative correlation between initial ammonium concentrations and nitrate reductions. This translates to low initial concentrations of ammonium inciting large uptake of nitrate. The significance of the correlation is supported by the small p-value (<0.05). The positive correlation coefficient for initial ammonium concentration and ammonium reductions suggest that increasing the initial concentration increases the reduction of ammonium. Otherwise, these results strongly support the nutrient uptake behaviors outlined in the literature.

Table 26. Correlation between ammonium, nitrate, and initial ammonium concentration.

Correlation Matrix - Pearson correlation/p-value		
	Ammonium	Nitrate
Nitrate	-0.409/0.001	
Ammonium Initial	0.482/0.000	-0.618/0.000

Nitrite reductions were much less consistent than ammonium and nitrate with regards to the various treatments. Since the algae cells have a preference to ammonium and nitrate the changes in nitrite might be attributable to bacterial contaminants conducting nitrification in the solution. The nitrite changes are indistinguishable for algae and bacteria and make analysis of the reductions difficult. The total phosphorus reductions shown in Table 25 appear to be inconsistent and depict both reductions and increases for the various treatments. This trend may be attributed to measuring total phosphorus instead of orthophosphate, as some studies suggest measuring. However, the reductions for total phosphorus seem to be primarily in the freshwater treatments while the increases are mostly for saltwater treatments.

The split plot ANOVA conducted on the various nutrients is displayed in Table 27 with each main effect and the interaction listed. The numbers reported are the corresponding p-values. Interestingly, for every nutrient except total phosphorus, the strain effect is not significant. This indicates that there is no significant difference between the strain treatments for total nitrogen, ammonium, nitrate, or nitrite. Alternatively, there is a significant difference between the wastewater concentrations for the ammonium, nitrate, and nitrite nutrients. Total nitrogen and total phosphorus did not

have significant differences for wastewater concentration. The results for the wastewater concentration effect were expected to have significant differences considering the premise of the experiment was that the wastewater was acting as a substitute nutrient source, therefore different levels of wastewater should portend different nutrient concentrations. However, it is interesting that the strain treatment effect was not significant for most of the nutrients.

Table 27. Test of fixed effects for split-plot ANOVA for nutrients (p-values are reported).

Effect	Total N	Ammonium	Nitrate	Nitrite	Total P
Strain	0.5310	0.4423	0.0878	0.9790	**0.0112**
Wastewater Concentration	0.5783	**0.0001**	**<.0001**	**<.0001**	0.1738

4.3.2 Regression Analysis of Initial Nutrient Concentrations and Growth Measurements

To obtain meaningful analysis of the nutrient data collected, it is useful to consider the impact of each nutrient and its available concentration on the growth of the microalgae. Using multiple linear regression, and the following equation (Eq. 4.1), the following analysis was conducted on each microalgae quantification method. The nutrient data used in the model was the initial concentrations available at the beginning of the growth period. Initially, all variables and their interactions were placed in the model and then were sequentially removed using backward stepwise elimination. When all variables remaining in the model were significant, the regression equation was accepted. The regression equations generated were placed in tables with each element separated for

clarity. Equations that resulted with a significant interaction term in the model were analyzed for each strain treatment separately to maintain model integrity. Eq. 4.1 is the original regression equation used for modeling the nutrient data.

$$\hat{y} = \beta_0\,(\text{Intercept}) + \beta_1(\text{Strain}) + \beta_2(\text{Total N}) + \beta_3(\text{Ammonium}) + \beta_4(\text{Nitrate}) + \beta_5(\text{Nitrite}) + \beta_6(\text{Total P}) + \varepsilon \quad (4.1)$$

Optical Density

The model generated for the optical density method resulted in strain, nitrate, total nitrogen, and the strain-total nitrogen interaction being significant. Because there was a significant interaction term in the model, four regression equations were generated for each strain treatment. Table 28 shows the four strain treatments with each element of the equation in the following columns. For example, based on Table 28, the regression equation for *Chlorella vulgaris* would be:

$$\text{Optical Density} = 1.08 - 0.01(\text{Total N}) + 0.03(\text{Nitrate})$$

The 95% confidence intervals (CI) included in Table 28 are corresponding to each of the significant elements within the equation and enable conclusions to be made about the population data, outside of the confines of this experiment. The combination of the regression equations and the confidence intervals allows conclusions to be made about the effects of these nutrients on the microalgae growth. For instance, with regards to the *Chlorella vulgaris* equation, a one unit increase in initial nitrate would correspond to

between 0.015 and 0.055 absorbance increase, with all other factors held constant. Alternatively, a one unit increase in initial total nitrogen would yield a change in absorbance from -0.022 to 0.003, with all other factors held constant.

The high R^2 values for the equations in Table 28 suggest that the model is very well represented by the elements included in it. The positive nitrate coefficients indicate that initial nitrate concentration has a positive relation to microalgae growth, which is consistent with the literature. However, in most of the equations the coefficient for total nitrogen includes zero in the confidence interval which does not support any conclusions being drawn about the impact of initial total nitrogen on microalgae growth.

Table 28. Regression equations for each strain treatment for optical density data.

Strain	Intercept	Total Nitrogen	Total Nitrogen 95% CI	Nitrate	Nitrate 95% CI	R^2
Chlorella vulgaris	1.08	-0.01	-0.022, 0.003	0.03	0.015, 0.055	0.98
Mixed Freshwater	1.22	-0.03	-0.060, 0.000	0.03	0.015, 0.055	0.93
Mixed Saltwater	0.14	0.03	0.012, 0.053	0.03	0.015, 0.055	0.86
Tetraselmis sp.	0.99	-0.02	-0.051, 0.002	0.03	0.015, 0.055	0.87

Cell Count

The backwards stepwise elimination used for building these models resulted in no significant explanatory variables. Therefore, no regression equations were generated based upon the cell count data. There are many possible explanations for this but it is most probably attributable to small sample size and high variation in the cell count data.

Algal Biomass by Chlorophyll-a Determination

The significant explanatory variables used in the model for the algal biomass data were initial ammonium and initial nitrate. The lack of a significant interaction allowed for only one regression equation to be generated for all the strain treatments. Table 29 displays the intercept and coefficients for the regression equation. However, this equation is limited due to the low R^2 value which indicates that there are external variables influencing the behavior of the algal biomass data. The interpretation of this regression equation states that a one unit increase in initial ammonium would yield a 1.339 to 4.027 unit increase in algal biomass, with all other factors held constant.

Albeit the poor R^2 for this regression equation, the findings are consistent with the suggestions in the literature. As expected, ammonium and nitrate are the most significant nutrients influencing microalgae growth. Also, for both initial ammonium and initial nitrate, the relationship is positively associated with growth. The inclusion of initial nitrate in the regression equation is also consistent with the regression equations derived for the optical density data. Similar to the problems with the cell count data, the low R^2 could potentially be attributable to high variability in the algal biomass data.

Table 29. Regression equations for algal biomass data.

Intercept	Ammonium	Ammonium 95% CI	Nitrate	Nitrate 95% CI	R^2
7.33	2.68	1.339, 4.027	2.81	1.146, 4.484	0.47

Volatile Suspended Solids

The significant explanatory variables included in the model for the volatile suspended solids data were strain, ammonium, nitrate, total nitrogen, and the strain-total nitrogen interaction. As was the case with the optical density data, an interaction term was significant in the model, and the resulting regression equations were separated by strain. Table 30 shows the intercept, coefficients, confidence intervals, and corresponding R^2 for the four strain treatments. The regression equation for the *Chlorella vulgaris* treatment is:

$$\text{Volatile suspended solids} = 513.36 - 9.35(\text{Total N}) + 10.9(\text{Ammonium}) + 18.22(\text{Nitrate})$$

This equation states that for a one unit increase in initial nitrate we could expect an increase of 18.22 mg/L VSS for our experimental data. However, when applying this equation outside of our experimental data, we are 95% confident we would expect between 2.37 and 34.07 unit increase in VSS for one unit increase in nitrate, with all other factors held constant. The corresponding R^2 for these equations are relatively large. As was the case with both the optical density and the algal biomass regression equations, nitrate remains a significant explanatory variable. These generated regression equations strongly support that nitrate and ammonium significantly influence microalgae growth, as

indicated by the literature. Another similarity of the equations in Table 30 to the equations derived for optical density is that the total nitrogen confidence intervals tend to include zero, making it difficult to infer any influences of initial total nitrogen on microalgae growth.

Based on the resulting regression equations, only optical density and volatile suspended solids yielded large coefficients of determination (R^2). These values suggest that a large proportion of the variation in the observed growth values can be attributed to the nutrients included in the model (Peck and Devore, 2008). Although the algal biomass method did yield a regression equation, the relatively low R^2 indicates a weakness in the utility of the model as defined. The cell count method was unable to return any significant strain or nutrient explanatory variables, therefore has no corresponding regression equation.

Table 30. Regression equations for each strain treatment for volatile suspended solids data.

Strain	Intercept	Total Nitrogen	Total Nitrogen 95% CI	Ammonium	Ammonium 95% CI	Nitrate	Nitrate 95% CI	R^2
Chlorella vulgaris	513.36	-9.35	-18.41, -0.287	10.9	0.786, 21.01	18.22	2.37, 34.07	0.93
Mixed Freshwater	365.60	-12.34	-24.14, -0.534	10.9	0.786, 21.01	18.22	2.37, 34.07	0.85
Mixed Saltwater	85.90	7.21	-3.862, 18.27	10.9	0.786, 21.01	18.22	2.37, 34.07	0.70
Tetraselmis sp.	606.77	1.45	-13.02, 15.93	10.9	0.786, 21.01	18.22	2.37, 34.07	0.89

4.4 Comparisons of Methods for Quantifying Algal Growth

Observing the error and limitations of each microalgae quantification method used in this study enables conclusions to be drawn about their relative effectiveness. For example, the coefficient of variation – a simple measure of variation in a method, standardized for comparison – enables easy comparison of these methods. The coefficient is defined by dividing the standard deviation (σ) by the mean (μ), resulting in a standardized number for variation. The larger the value, the greater the variation observed in the method. Figure 31 displays the coefficient of variation for each method used for quantifying the microalgae growth. The algal biomass by chlorophyll-a determination had the largest coefficient, and the optical density method had the smallest value.

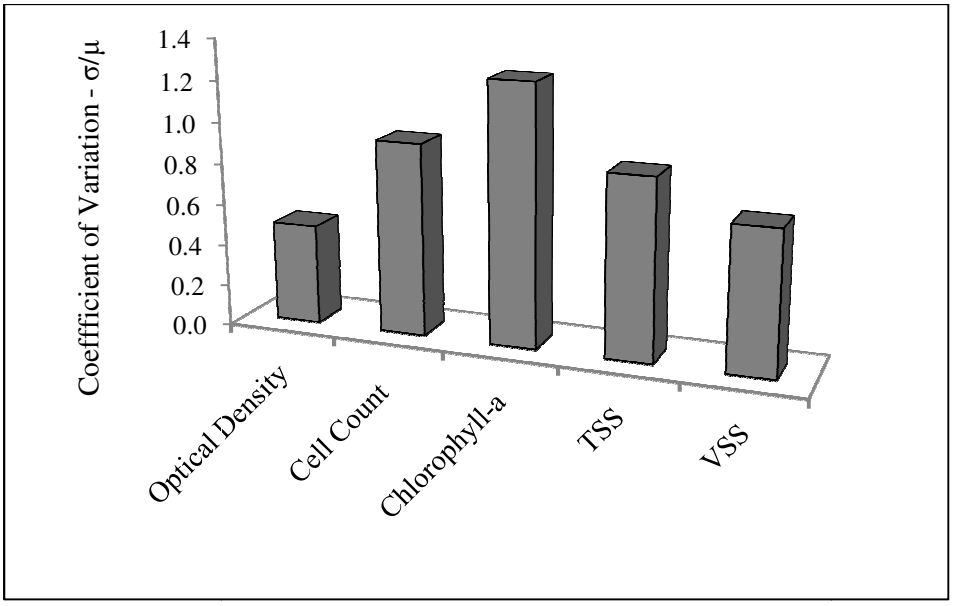


Figure 31. Comparison of the analytical methods used in this study.

Hypothesis testing for method comparisons by correlation:

Null hypothesis (H_o): there is no correlation between the methods ($\rho = 0$)

Alternative (H_a): there is a correlation between the methods ($\rho \neq 0$)

In addition to the coefficient of variation, it was useful to examine the correlations between these methods to determine the adequacy of each method as compared to the alternatives. Table 31 shows the Pearson correlation coefficient and p-value for each method in comparison to all other methods. The second value – the p-value – is the statistical value that determines if the correlation in question is significant. If the p-value was significant, we rejected the null hypothesis and concluded that there is a correlation between the corresponding methods. The bold numbers (p-value < 0.05) indicate a statistically significant correlation between the corresponding methods. A high correlation between methods suggests that one method could serve as a surrogate for the other, thereby eliminating the need for excessive laboratory work. Additionally, the correlations highlight discrepancies between analytical methods and further showcase the need for multiple methods to effectively quantify microalgae in wastewater growth media. Note that the cell count values included contaminants; otherwise, the observed cell counts would be artificially lower than the biomass observations by other methods.

Table 31. Correlation coefficients for analytical methods employed in this study.

Correlation Matrix - Pearson correlation/p-value				
	Cell Count	Chlorophyll-a	TSS	VSS
Chlorophyll-a	0.199/0.052			
TSS	0.183/0.109	0.204/0.073		
VSS	0.082/0.473	0.303/**0.007**	0.882/**0.000**	
Optical Density	0.519/**0.000**	0.113/0.475	0.753/**0.000**	0.778/**0.000**

Table 31 demonstrates that optical density measurements are significantly correlated to cell count, total suspended solids, and volatile suspended solids. Considering the substantially lower time requirement for optical density measurements, these results suggest that optical density could potentially negate the requirement to measure cell count and solids. The most challenging measurement, as well as highly variable (Fig. 31), was the chlorophyll-a determination for algal biomass. This method was not strongly correlated to any other method except for the volatile suspended solids method. While this method is a recognized scientific method, it appears to have low utility for measuring microalgae growth in wastewater media. The cell count correlation coefficients also were insignificant for all methods except for optical density. However, the advantages of conducting cell counts; including contaminant detection and visual quantification, outweigh the shortcomings. Additionally, it is important to consider the human error associated with this method, and the fact that it was strongly correlated to the useful optical density method. The volatile suspended solids method also exhibited valuable correlation to optical density measurements and maintained fairly low variability. As compared to cell counts and absorbance, the major advantage of volatile

suspended solids measurements is to obtain dry weight and density values for algal biomass.

Another useful tool to assess the efficacy of the various methods is to observe the experimental data analysis for the microalgae growth and regression analysis for the nutrients. The split plot ANOVA conducted for microalgae growth found that optical density and volatile suspended solids yielded the highest levels of significance and ultimately provided the most refined results. The significant interactions for those two methods could be attributable to greater method precision and lower experimental error. The regression analysis for nutrients supported the utility of the optical density and volatile suspended solids methods by resulting in relatively high R^2 values for the corresponding regression equations. While those two methods had R^2 values above 0.7, the algal biomass method was 0.47, and the cell count method could not even determine any significant explanatory variables. Overall, with the analysis methods considered, as well as the coefficient of variation and correlation test, the two strongest methods were optical density and volatile suspended solids.

CHAPTER V

CONCLUSIONS

5.1 Experimental Conclusions

The experimental results of this study can be analyzed in many different levels of detail with some challenging interpretations. Ultimately the three specific objectives were to observe microalgae growth at different levels of wastewater concentration and with different strain treatments. During the process of measuring the growth, various common microalgae quantification methods were to be compared for their relative strengths and weaknesses for these conditions. Additionally, some select nutrients were to be observed for their uptake rates and the influences they might have on growth. These three specific objectives are the organizational basis for which conclusions will be drawn.

The comparison of the microalgae quantification methods assessed how precise the methods were during this experiment, and how they compared to one another. Based upon the coefficient of variation and the correlation analysis, the optical density and volatile suspended solids tests are the most appropriate measures of microalgae growth under these experimental conditions. The cell count method was hindered by the high variability in the observed values, which can potentially be attributed to the significant human operator bias and error in that method. Additionally, the optical density and volatile suspended solids methods produced valuable regression equations based on the nutrient observations as explanatory variables. Based upon these consistencies, the

volatile suspended solids and optical density methods were most heavily utilized in formulating overall conclusions.

5.1.1 Microalgae Growth

Although this experiment was hindered by some experimental error some major conclusions can still be reached. Microalgae growth was maximized at the 25% and 33% wastewater concentrations with limited difference from the control. This effectively concludes that those concentrations of wastewater can be successfully utilized in place of the traditional nutrition provided by generic plant foods. This verifies the great opportunity for commercial scale cultivation of microalgae utilizing wastewater media to reduce nutrition costs. Obviously these concentrations serve only as preliminary guidelines for the operational quantities of wastewater for maximum biomass production.

The *Chlorella vulgaris* and *Tetraselmis sp.* strains demonstrated strong growth consistently under the wastewater conditions of this experiment. Despite contamination and biological competition concerns, these microalgae strains proved viable for cultivation. However, the statistical analysis of this experiment generated mixed results about whether these strains significantly out-competed the mixed culture treatments. Regardless, the strain factor was relegated when considered simultaneously with wastewater concentration. If commercial cultivation requires pure culture production of microalgae there will be much greater emphasis and sterilization and wastewater will likely be an unacceptable option. For the purposes of producing biofuels microalgae biomass must be maximized, with a lesser concern on culture purity and contamination.

Based on the results generated from the strongest microalgae quantification method, the volatile suspended solids method, a few general conclusions can be made. From the Tukey comparisons in Table 21, the treatments with the highest mean volatile suspended solids were the *Tetraselmis* 0%, 33%, 25%, and *Chlorella* 33%, 25%, and 10% respectively. The wastewater treatments for the *Chlorella* treatments were significantly greater than their corresponding control. This means that the wastewater treatments actually yielded greater algal growth than the plant food for *Chorella vulgaris*. For the *Tetraselmis* treatment, the control was not significantly greater than the wastewater treatments so the 25% and 33% wastewater treatments can be considered effective substitutes for plant food.

5.1.2 Nutrient Observation and Effect upon Microalgae Growth

The results of the nutrient analysis supported the literature's suggestion that ammonium and nitrate would have very significant effects upon the growth of microalgae. Ammonium and nitrate commonly had the largest reductions and they were also repeatedly significant in the regression equations used for predicting microalgae growth. Based upon the results from the wastewater treatments, it is reasonable to conclude that the nutrient composition of the dairy wastewater is suitable for microalgae growth. However, for a more nutrient-specific breakdown a more technical and focused study would need to be conducted on the behavior of the aforementioned, as well as including many more primary and secondary plant nutrients.

The change of nutrient concentration during the experimental growth period indicated that achieving huge reductions for pollution considerations are likely not synonymous with maximizing microalgae biomass. Although reductions are a goal, the

nutrient concentrations must remain at levels desirable for the best exponential growth of the microalgae. This experiment did obtain very high nitrate reductions, which might indicate a nitrate deficiency that hindered optimum biomass growth. Also, the nitrate reductions were unrelated to the wastewater concentration, with approximately equal reductions for all treatments except the control. Regarding the utility of microalgae growth for remediation of agricultural waste streams, the significant nitrate reductions are very encouraging. Such pollutants require expensive treatment to meet regulations, and incorporating microalgae for partial treatment appears to have potential. Although the other nutrient reductions were inconsistent compared to nitrate, there are positive implications for microalgae and wastewater remediation. For commercial scale operations, nutrient concentrations should be continuously monitored and managed to optimize the growing environment for the microalgae. Clearly, the concentration of nitrate is vital to successful cultivation of biomass.

The regression analysis emphasized the importance of nitrate and ammonium as predictors for microalgae growth. Most of the equations derived included at least one of nitrate or ammonium, especially amongst the equations with high R^2 values. Additionally, the relationship between nitrate and ammonium with growth was always positive; suggesting an increase in either nutrient would result in increased biomass yield. Ultimately, the regression analysis provides a theoretical capability to predict biomass from available nutrients, but more likely emphasizes the importance of those nutrients for microalgae growth.

5.1.3 Microalgae Quantification Methods

Summarizing the results of the split-plot ANOVA on biomass growth, the regression analysis of nutrients and the method comparisons strongly suggests which methods were most effective. While better funded and staffed laboratories could successfully implement all of these methods, or possibly more advanced methods, a combination of optical density and volatile suspended solids would be highly recommended for further experiments under similar experimental conditions. Additionally, periodic cell counts are necessary to assess sample purity and population dynamics. Ultimately, a well executed combination of optical density and volatile suspended solids can yield both fast, reliable laboratory results and tangible biomass quantification units.

5.2 Future Research

This study contributes a small piece of scientific information to the large intellectual pool that can help to bring microalgae to reality as a biofuel feedstock. Much research can still be done to reduce the costs of producing microalgae and processing it into usable fuel and bioproducts. However, to truly reach a commercial scale for microalgae grown in wastewater media, there are a few major areas that need to be investigated. Primarily, further research must be at a commercial scale to provide figures for what quantities of wastewater will maximize biomass produced and minimize pollutants in the effluent. This research could be conducted in commercial scale photobioreactors or small scale chemostats. Ideally, the specific constituents within the wastewater could be closely observed at more frequent time intervals rather than analysis at the beginning and end of the growth period. This type of experiment could translate

into recipes for maximizing microalgae growth and optimizing nutrient dosing at the commercial scale.

Regarding laboratory scale experiments there are numerous opportunities to improve accuracy and precision of the results. Experimental designs in these microbiological fields require larger replication for greater statistical power. Although difficult and costly, this can be executed by reducing experimental treatments and increasing repetition to achieve larger sample sizes. Additionally, special attention must be paid to maintaining rigid aseptic equipment and techniques during laboratory experiments. This includes substantial preparation and planning and can also be improved by conducting preliminary test studies. Another tremendous lesson from this experiment is to maintain narrow research parameters and focus on statistical power rather than a large scope.

This experiment offers tremendous lessons and provides a foundation for further research in utilizing wastewater for microalgae cultivation. The uncertainty about methods is clarified here by including many methods and assessing their effectiveness. Future work should include fewer methods, ideally utilizing the best performing methods as concluded in this study. The narrower research goals and fewer laboratory methods will ensure that potential research will have strong statistical integrity and a high ability to detect the subtle differences that can greatly influence a commercial scale operation.

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Appendix A: Cell Counting Method

Adapted from Standard Methods, method 10200E and 10200F (APHA, 1998)

1. Collect a uniform sample of 5mL. Make sure that sample source is well mixed and receptacle is clean. Label receptacle accordingly.
2. Turn on microscope and make sure the 4X objective is in position.
3. Make sure hemocytometer and cover slip (special type) are cleaned with a lens paper, not kim wipes (they will scratch glass). Place hemocytometer cover slip over counting chamber so that is reaches across the entire width of the hemocytometer.
4. Wash Pasteur pipet with distilled water by sucking up a few milliliters and dumping it.
5. While drawing sample into Pasteur pipet, make sure that sample is thoroughly mixed and pull sample from outside of the vortex (the center of the mixing sample).
6. Return the first few drops back into the sample to remove air bubbles.
7. Add enough sample into the counting chamber, beneath the coverslip, to fill the entire counting chamber without exceeding the boundaries (if too much is added, the hemocytometer and the cover slip must be re-washed with distilled water).
8. Fill both sides of the counting chamber.
9. Place slide under microscope and focus in on one side of the counting chamber under 4X.
10. Once focused, move the objectives to the 20X or 40X (careful not to break cover slip under lens). Re focus.
11. Find grid with smallest graduations (should be the 25 centermost squares).
12. Starting with the lower left square (see below) count the cells within it. Cells touching the left and bottom boundary are not included, and cells touching the top and right edge are included.
13. Continue the counting on the upper left, upper right, lower right, and center square.
14. Sum the total of the squares and perform the following calculation:

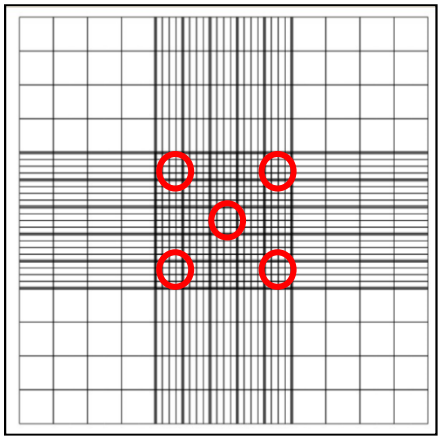

Figure 32. Hemocytometer counting grid with cells counted encircled. Source: After Kim, 2010.

$$\textbf{cells per mL} = \frac{(\textbf{total cells in five squares})\text{x}(1000)}{0.02}$$

Appendix B: Total Suspended Solids and Volatile Solids

Adapted from Standard Methods, method 2540 (APHA, 1998):

Filter Preparation: All filters must be pre-dried and weighed prior to use.

1. Prepare 18 (or appropriate number) of G4 glass fiber filters by rinsing over vacuum with distilled water.
2. Vacuum until all visible water is removed.
3. Place each filter in its corresponding crucible and place in furnace (put all filters and crucibles into the furnace simultaneously) at 550 ^{0}C for 15 minutes.
4. Remove from furnace and place sampels in bell jar dessicator(not dessicator cabinet, it will melt at high temps). Wait for samples to cool to room temp before handling.

Total Suspended Solids (TSS): Each sample will be measured using triplicates.

5. Collect uniform sample of 10 mL from well mixed solution. Use clean beaker for each sample and label accordingly.
6. Place first filter on filter apparatus and moisten with distilled water. Turn on pump.
7. Add 2 mL of sample to center of filter. Follow with a few milliliters of distilled water to thoroughly filter all small particles. Once sample is visibly dry, stop pump and replace filter with its corresponding crucible.
8. Repeat process for all replicates of all samples.
9. Place all samples in oven at 103-105^{0}C for 1 hr.
10. Remove samples and cool in dessicator cabinet for a few minutes. Remove each sample individually and measure weight. (sample does not have to be returned to dessicator once weight has been recorded).

Volatile Solids (VS): Samples will be ashed at 550^0 C for 1 hr to determine organic content.

11. Using same samples from TSS method; place crucible, filter, and accumulation into furnace at 550^0 C for 1 hr (all at the same time).
12. Remove samples and place directly into bell jar dessicator to cool. Allow time to cool to room temp.
13. Remove samples individually and immediately record weight (they will take on atmospheric moisture over time).

Calculations:

After procedure, the following values are obtained:

A = Dry filter and crucible weight, g

B = Dry weight of residue, filter and crucible, g

C = Ash weight of residue, filter and crucible, g

V = Volume of sample, mL

$$\text{TSS, mg/L} = \left(\frac{(B-A)}{V}\right)\left(\frac{1000\ mg}{1\ g}\right)\left(\frac{1000\ mL}{1\ L}\right)$$

$$\text{VS, mg/L} = \left(\frac{(B-C)}{V}\right)\left(\frac{1000\ mg}{1\ g}\right)\left(\frac{1000\ mL}{1\ L}\right)$$

Appendix C: Algal Biomass by Chlorophyll-a Determination

Adapted from Standard Methods, method 10200 H (APHA, 1998):

Preparation of Aqueous Acetone Solution:

1. Add 1.0 g finely powdered $MgCO_3$ to 100 mL of distilled water and mix.
2. Mix 90 parts (900 mL) reagent grade acetone with 10 parts (100 mL) saturated magnesium carbonate solution, mix.

Sample Concentration and Extraction: (all work must be conducted in subdued light to avoid error)

1. Collect a well-mixed sample and place in subdued light if storage is necessary.
2. Pipet 15 mL of sample into screw-cap graduated centrifuge tubes.
3. Centrifuge samples at 1000g for 15 minutes.
4. Remove supernatant water using a pipet.
5. Rinse extracted slurry with acetone solution 3 times into tissue grinder.
6. Grind solution for 2 minutes.
7. Rinse macerated solution back into clean labeled 15 mL centrifuge tubes with acetone solution 2 or 3 times.
8. Fill volume in centrifuge tube with acetone solution to a final volume of 15 mL.
9. Store sample for 24 hr in dark refrigerator (4 deg C).

Measuring Chlorophyll-a content:

1. Remove samples from refrigerator and place in centrifuge. Centrifuge at 500 g for 20 minutes.
2. Transfer 3 mL of clarified extract (supernatant) into a 1 cm cuvette.
3. Read optical density (OD) at 750 and 664 nm.
4. Acidify extract with 0.1 mL of 0.1N HCl. Wait 90 seconds.
5. Read OD at 750 and 665 nm.

Calculations:

1. Subtract the OD values at 750 nm from the 665 nm and 664 nm values respectively.
2. Calculate the chlorophyll-a content, convert to appropriate units if necessary.
3. Use the multiplier to convert from chlorophyll-a to algal biomass.

$$\text{Chlorophyll-a, mg/m}^3 = \frac{26.7\,(664b - 665a)\,x\,V1}{V2\,x\,L}$$

V_1 = volume of extract, L

V_2 = volume of sample, m^3

L = pathlength of cuvette, cm

664_b, 665_a = optical densities before and after acidification, respectively.

Algal Biomass, mg/m^3 = chlorophyll-a content (mg/m^3) x 67